Volume 23 January 1959 No. 1

BULLETIN OF THE AGRICULTURAL CHEMICAL SOCIETY OF JAPAN

ANALYTICAL CHEMISTRY ORGANIC CHEMISTRY BIOLOGICAL CHEMISTRY
FOOD & NUTRITION MICROBIOLOGY & FERMENTATION INDUSTRY
SOIL & FERTILIZER

CONTENTS

Organic Chemistry

M. MATSUI and Y. NISHIZAWA: Attempted Syntheses of Tetracyclin Analogues. Part I. 1
K. IDA: Polarographic Studies on Sarkomycin. Part I. Polarography of Sarkomycin, its Degradation or Polymerization Products, and 3-Methyl-2-oxocyclopentane-1-carboxylic Acid. 40

Biological Chemistry

D. FUKUSHIMA: Studies on Soybean Proteins. Part I. Water Dispersibility of Protein of Defatted Soybean Flour as a Criterion for Degree of Denaturation. .. 7
D. FUKUSHIMA: Studies on Soybean Proteins. Part II. A New Method for Quantitative Determination of the Degree of Denaturation of Protein in Soybean Flour. 15
Y. MORITA and K. KAMEDA: Studies on Phyto-peroxidase. Part XII. Amino Acid Composition of Japanese-radish Peroxidase a. 28
M. ITO and S. YOSHIDA: Determination of Iodine Affinity of Starch by Amperometric Titration. 34

Food and Nutrition

T. KOYANAGI, S. HAREYAMA and M. OTA: The Effect of Riboflavin on Hypertension induced by High Salt Diet in the Rat. 3

Microbiology and Fermentation Industry

K. SAKAGUCHI: Studies on the Activities of Bacteria in Soy Sauce Brewing. Part IV. Proteases, Transaminases of *Pediococcus soyae* nov. sp., and its Numerical Transition in Soy Mash. 22
K. MORIHARA: Studies on the Protease of *Pseudomonas*. Part IV. Factors Affecting the Enzyme Production, especially on the Effect of Calcium. 49
K. MORIHARA: Studies on the Protease of *Pseudomonas*. Part V. On the Role of Calcium in the Enzyme Production. 60

(continued on third page of cover)

Published by the Agricultural Chemical Society of Japan
c/o Faculty of Agriculture, University of Tokyo

THE AGRICULTURAL CHEMICAL SOCIETY OF JAPAN
(NIPPON NÔGEI-KAGAKU KAI)

President
Sankichi TAKEI

Vice Presidents
Takeo KUMAZAWA　Makoto KANDATSU

EDITORIAL BOARD

Chairman: Kin-ichirô SAKAGUCHI

Hisashi ARIYAMA
Toshinobu ASAI
Fujio EGAMI
Yoshiyuki INOUE
Hisayoshi IWATA
Hideo KATAGIRI
Nobuhiko MIGITA
Ziro NIKUNI
Yatarô OBATA
Azuma OKUDA
Yasuyoshi ÔSHIMA
Yoshito SAKURAI
Rinjirô SASAKI
Toriji SASAKI
Yusuke SUMIKI
Isamu TACHI
Sankichi TAKEI
Teiichi TAMURA
Izue YAMASAKI

Advisory Editors:
Kakuji GOTÔ
Kinsuke KONDÔ
Teijirô YABUTA
Yuzuru OKUDA

Managing Editors:
Saburo FUNAHASHI　Yônosuke IKEDA

The Bulletin of the Agricultural Chemical Society of Japan is published bimonthly to provide original papers on agricultural chemistry.

Manuscripts submitted for publication and all inquiries regarding them should be sent to the Editor of the Bulletin, The Agricultural Chemical Society of Japan, c/o Departments of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo, Bunkyo-ku, Tokyo, Japan.

Annual subscription price is $6.00 for all oversea subscribers including postage fee. Please address order to the agencies.

The Agricultural Chemical Society of Japan also published The Journal of the Agricultural Chemical Society of Japan (Nippon Nôgei-Kagaku Kaishi), printed in Japanese.

AGENCIES

Charles E. Tuttle Co.
Rutlant, Vt.
U.S.A.

Maruzen Co., Ltd.
P. O. Box 605, Tokyo Central
Tokyo, Japan.

BULLETIN OF THE AGRICULTURAL CHEMICAL SOCIETY OF JAPAN

ANALYTICAL CHEMISTRY ORGANIC CHEMISTRY BIOLOGICAL CHEMISTRY
FOOD & NUTRITION MICROBIOLOGY & FERMENTATION INDUSTRY
SOIL & FERTILIZER

Volume 23

1959

EDITORIAL BOARD
Chairman: Kin-ichirô SAKAGUCHI
Hisashi ARIYAMA Toshinobu ASAI Fujio EGAMI
Yoshiyuki INOUE Hisayoshi IWATA Hideo KATAGIRI
Nobuhiko MIGITA Zirô NIKUNI Yatarô OBATA
Azuma OKUDA Yasuyoshi ÔSHIMA Yoshito SAKURAI
Rinjirô SASAKI Toriji SASAKI Yusuke SUMIKI
Isamu TACHI Sankichi TAKEI Teiichi TAMURA
Izue YAMASAKI

Advisory Editors:
Kakuji GOTÔ Kinsuke KONDÔ Yuzuru OKUDA
Teijirô YABUTA

Published by the Agricultural Chemical Society of Japan
c/o Faculty of Agriculture, University of Tokyo

CONTENTS OF VOLUME 23, 1959

No. 1

Organic Chemistry

M. MATSUI and Y. NISHIZAWA: Attempted Syntheses of Tetracyclin Analogues. Part I....... 1

K. IDA: Polarographic Studies on Sarkomycin. Part I. Polarography of Sarkomycin, its Degradation or Polymerization Products, and 3-Methyl-2-oxocyclopentane-1-carboxylic Acid... 40

Biological Chemistry

D. FUKUSHIMA: Studies on Soybean Proteins. Part I. Water Dispersibility of Protein of Defatted Soybean Flour as a Criterion for Degree of Denaturation......................... 7

D. FUKUSHIMA: Studies on Soybean Proteins. Part II. A New Method for Quantitative Determination of the Degree of Denaturation of Protein in Soybean Flour. 15

Y. MORITA and K. KAMEDA: Studies on Phyto-peroxidase. Part XII. Amino Acid Composition of Japanese-radish Peroxidase a. 28

M. ITO and S. YOSHIDA: Determination of Iodine Affinity of Starch by Amperometric Titration... 34

Food and Nutrition

T. KOYANAGI, S. HAREYAMA and M. OTA: The Effect of Riboflavin on Hypertension induced by High Salt Diet in the Rat.. 3

Microbiology and Fermentation Industry

K. SAKAGUCHI: Studies on the Activities on Bacteria in Soy Sauce Brewing. Part IV. Proteases, Transaminases of *Pediococcus soyae* nov. sp., and its Numerical Transition in Soy Mash. 22

K. MORIHARA: Studies on the Protease of *Pseudomonas*. Part IV. Factors Affecting the Enzyme Production, especially on the Effect of Calcium. 49

K. MORIHARA: Studies on the Protease of *Pseudomonas*. Part V. On the Role of Calcium in the Enzyme Production. 60

Soil and Fertilizer, Inorganic Chemistry

M. NAKAYAMA: A New Basic Triple Salt Containing Magnesium Hydroxide. Part II. The Quaternary System KCl–MgCl$_2$–Mg(OH)$_2$–H$_2$O at 100°. 46

Short Communications

K. MUNAKATA, T. SAITO, S. OGAWA and S. ISHII: Oryzanone, an Attractant of the Rice Stem Borer. 64

S. NAKAMURA and T. SHIRO: Hydroxyaspergillic Acid as Growth Inhibitant against Hiochi-bacteria (Studies on Growth Inhibition of Hiochi-bacteria, Specific Saprophytes of Saké. Part 4). 65

K. ANNO, N. SENO, E. NAKAMURA, H. SAITO and R. HOSHI: Isolation of Laminaribiose from Reversion Products of D-Glucose. 67

T. YAMAMOTO and J. FUKUMOTO: Reactivation of Bacterial α-Amylase Inactivated with Ethylenediamine Tetraacetate. 68

K. YAMADA, T. SUZUKI and M. TANAKA: Decomposition and Formation of Oxalate in Fish Skeletal Muscle. 71

No. 2

Analytical Chemistry

K. SAKAMOTO, K. SAITO and A. NAGATA: On the Migration Distance in Paper-electrophoresis. Part VII. Studies on the Migration Distance of Dyes. (2). 89

K. SAKAMOTO, K. SAITO and A. NAGATA: On the Migration Distance in Paper-electrophoresis. Part VIII. On the MG Values of Dyes. 95

Organic Chemistry

Y. OBATA and J. MIZUTANI: Isolation and Identification of L-Isoleucyl-L-valine Anhydride from the Soy-bean Meal Hydrolysates (AMINOSAN-EKI). 121

R. TAKEDA: *Pseudomonas* Pigments. III. Derivatives of Pyoluteorin. 126

Biological Chemistry

H. OMURA and H. TAKAHASHI: On the Nitrate Reductase of Cattle Liver. 83

Y. OBATA and M. KOSHIKA: Effect of Ultraviolet Light Irradiation on the Peptide Bonds of Protein. 107

A. KAJI: Studies of Macerating Enzyme acting on Middle Lamella Pectin. Part IV. Action of Macerating Enzyme on Pectic Substances. 131

Food and Nutrition

K. MORI and Y. NAKAMURA: Studies on the Metabolism of Lipids. Part I. Influence of Fat-deficiency on Body Fat. 78

S. SAKAMURA and Y. OBATA: Comparative Studies of Brown Discoloration and Ammonia Liberation from the Milk Proteins by Ultraviolet Light Irradiation. 116

Microbiology and Fermentation Industry

W. WATANABE: Effect of Gamma-ray upon Food Microorganisms. (Studies concerning Gamma-ray Resistivity of *Escherichia coli*. III). 73

K. SAKAGUCHI: Studies on the Activities of Bacteria in Soy Sauce Brewing. Part V. The Effects of *Aspergillus sojae*, *Pediococcus soyae*, *Bacillus subtilis* and *Saccharomyces rouxii* in Purely Cultured Soy Sauce Brewing. 100

K. YAMAMOTO: Studies on Koji. Part IV. Mold Protease Production by Replacement Culture and Inactive Form Protease in Cell Free Extract. 110

Short Communications

T. HASEGAWA and T. TAKAHASHI: Microbiological Oxidations of Reichstein's Compound S by *Corticium solani*. 137

S. HIZUKURI: Formation of Native Starch-like Granules of Amylodextrin in Vitro............ 138
Y. HIROSE and T. NAKATSUKA: Terpenoids. Part IV. Further Investigation on the Constitution of Occidentalol. 140
M. MIYANO und M. MATSUI: Synthesen und Konfigurationsermittlung in der Rotenoid-Reihe, IX. Synthese des Roteols und Hydroxyroteols. 141

No. 3

Organic Chemistry

Y. HIROSE and T. NAKATSUKA: Terpenoids. Part IV. The Structure of Occidol, a New Sesquiterpene Alcohol from *Thuja occidentalis* L. 143
R. TAKEDA: *Pseudomonas* Pigments. IV. The Structure of Pyoluteorin............ 165
T. ISHIHARA and T. KONDO: Studies on Lignin. Part VI. Hydrogen Sulfide Cooking and Subsequent Alkali Treatment of Guaiacylpropanediols and Several Other Lignin Model Compounds. 178
M. IZAWA, Y. KOBASHI and S. SAKAGUCHI: Phenolic Components in Cigarette Smoke. 194
M. IZAWA, Y. KOBASHI and M. TAKI: Free Amino Acids in Cigarette Smoke. (I). 198
M. IZAWA and M. TAKI: Free Amino Acids in Cigarette Smoke. (II). 201
K. YAGISHITA: The Chemistry of Ilexol. IV. The Identity of Acetate with Urs-13(18)-en-3β-yl Acetate together with the Formation of Isoilexone, Isoilexenedione and Isoilexene. 217
K. YAMASHITA and M. MATSUI: Studies on Phenolic Lactones. Part II. A New Synthetic Method of Isohibalactone. 230
T. KONDO, H. IMAMURA and M. SUDA: Wood Extractives. Part VIII. On the Heartwood Constituents of *Cryptomeria japonica* D. Don. (1) 233

Biological Chemistry

Y. SATOMURA, S. OI, A. SAWADA and J. FUKUMOTO: Biochemical Investigations on the Lipase Formation of *Sclerotinia* Fungus and on the Specificity of Lipase Action of Some Fungal Origins. 150
S. SUGAWARA, Y. NAKAMURA and T. SHIMOMURA: Fractionation of Maltase and Saccharogenic Amylase in Mold, and Crystallization of Maltase. 156
M. HAYASHI and S. SHICHIJI: Effects of 2,4-Dinitrophenol on Endogenous Respiration of Yeast harvested during the First Budding Cycle............ 159
K. KATSUDA, T. CHIKAMOTO and Y. INOUYE: Relationship between Stereoisomerism and Biological Activity of Pyrethroids. Part IV. The Oxidative Degradation of (±)-Allethrolone-methylester. 171
Y. KATSUDA, T. CHIKAMOTO and Y. INOUYE: Relationship between Stereoisomerism and Biological Activity of Pyrethroids. Part V. The Absolute Configuration of (+)-Pyrethrolone and (+)-Cinerolone. 174
H. CHIBA and E. SUGIMOTO: Studies on Crystalline Yeast Phosphoglyceric Acid Mutase. I. Basic Properties and Effects of Several Inhibitors. 207
H. CHIBA and E. SUGIMOTO: Studies on Crystalline Yeast Phosphoglyceric Acid Mutase. II. Electrophoretic Separation of Each Component. 213

Microbiology and Fermentation Industry

Y. SATOMURA, S. OKADA and J. FUKUMOTO: Action of Lysozyme and Some Microbial Enzymes on Acid-Resistant Organism. (Studies on Bacteriolytic Substances produced by Microorganisms. Part 5). 145

Y. OBATA and H. HORITSU: Studies on the Sunlight Flavour of Beer. Part VI. Correlation of the Occurrence of the Sunlight Flavour of Beer to Humulone, Lupulone and Related Compounds. 186

T. HARADA: Effect of Carbon and Nitrogen Sources on the Utilization of Tyramine and Phenolsulphatase Biosynthesis by Cell Suspentions of *Aerobacter aerogenes*. 222

A. KUNINAKA, S. OTSUKA, Y. KOBAYASHI and K. SAKAGUCHI: Studies on 5′-Phosphodiesterases in Microorganisms. Part I. Formation of Nucleoside-5′-monophosphates from Yeast Ribonucleic Acid by *Penicillium citrinum*. 239

Short Communications

M. SHIRASAKA, R. HAYASHI and M. TSURUTA: Microbiological Hydroxylation of Steroids by *Absidia regnieri*. 244

M. SHIRASAKA, R. TAKASAKI, R. HAYASHI and M. TSURUTA: Microbiological Hydroxylation of Progesterone and 17α-Hydroxyprogesterone by *Sclerotinia libertiana*; 2β-Hydroxylation... 245

M. ABE, T. YAMANO, S. YAMATODANI, Y. KŌZU, M. KUSUMOTO, H. KOMATSU and S. YAMADA: On the New Peptide-Type Ergot Alkaloids, Ergosecaline and Ergosecalinine. 246

No. 4

Analytical Chemistry

K. MORI and M. NAKAMURA: Notes on the Colorimetric Determination of Inorganic Orthophosphate. Part I. Determination of Inorganic Orthophosphate in the Presence of Some Acid-labile Phosphate Compounds of Biochemical Significance. 272

K. MORI and M. NAKAMURA: Notes on the Colorimetric Determination of Inorganic Orthophosphate. Part II. Determination of Total Phosphorus. 278

Organic Chemistry

Y. HIROSE and T. NAKATSUKA: Terpenoids. Part V. The Synthesis of Occidol. 253

H. WATANABE: The Chemical Structure of the Intermediate Metabolites of Catechin. I. Chemical Properties of the Intermediate Metabolites (G and H) and their Derivatives. 257

H. WATANABE: The Chemical Structure of the Intermediate Metabolites of Catechin. II. Oxidative Decomposition of the Intermediate Metabolites (G and H). 260

H. WATANABE: The Chemical Structure of the Intermediate Metabolites of Catechin. III. Synthesis of Intermediate Metabolites (G and H). 263

H. WATANABE: The Chemical Structure of the Intermediate Metabolites of Catechin. IV. Structure of the Intermediate Metabolite (F). 268

H. OGAWA, T. ITO, S. KONDO and S. INOUE: The Structure of an Antibiotic Kanamycin. .. 289

Biological Chemistry

K. TAKAHASHI and Y. KONDO: The Formation of L-Glutamic Acid from α-Ketoglutaric Acid by a Constructed Enzyme System. 249

A. KUNINAKA: Studies on the Decomposition of Nucleic Acid by Microorganisms. Part VI. On the Ribosidase of *Aspergillus oryzae* Acting on 6-Hydroxypurine Ribonucleosides and their 5′-Monophosphates. 281

Microbiology and Fermentation Industry

K. IZAKI: Studies on the Metabolism of D-Amino Acid in Microorganisms. Part VII. Possible Involvement of Cell Permeability in the Metabolism of L-Glutamic Acid by *Aerobacter aerogenes* Strain A. 310

M. TAKAKUWA: Studies on Dry Yeast. (IV). Mechanism of Fermentation of Dry Bakers' Yeast. 316

H. KATAGIRI, T. TOCHIKURA and Y. SUZUKI: Microbiological Studies of *Coli-aerogenes* Bacteria. Part VI. The Action of Antibiotics on Bacterial Respiration and α-Ketoglutaric Acid-Fermentation. 322

H. ÔNISHI: Studies on Osmophilic Yeasts. Part IV. Change in Permeability of Cell Membranes of the Osmophilic Yeasts and Maintenance of their Viability in the Saline Medium. .. 332

Short Communications

H. CHIBA, E. SUGIMOTO and M. KITO: Separation and Crystallization of Each Component from Crystalline Phosphoglyceric Acid Mutase. 340

T. ISHIHARA and T. KONDO: Studies on Lignin. Part VII. Isolation and Identification of 2-Ethylthio-1-(4-hydroxy-3-methoxyphenyl)-propanone-(1) from Mercaptolysis Oil of Pine Ethanol Lignin. 341

T. WATANABA and K. ASO: Isolation of Isomaltose from Honey. 342

A. KAWARADA and Y. SUMIKI: The Occurrence of Gibberellin A_1 in Water Sprouts of Citrus. 343

H. KITAMURA, N. TAKAHASHI, Y. SETA, A. KAWARADA and Y. SUMIKI: Biochemical Studies on "Bakanae" Fungus. Part 54. Chemical Structure of Gibberellins. Part XX. 344

K. SASAOKA: Occurrence of Homoserine Dehydrogenase in Pea Seedlings. 347

K. NAKAMURA and H. ARIYAMA: The Preventive Effect of a S-Methyl Methionine Sulfonium Compound on Dietary Hypercholesteremia. 348

No. 5

Analytical Chemistry

K. MORI and M. NAKAMURA: Quantitative Analysis of Sugars in Plant Extracts by Ion-exchange Chromatography, with Special Reference to the Examination of Conditions for preparing the Sample Sugar Solutions. 389

Organic Chemistry

H. UEDA, Y. SHIBAHARA and S. SHIMIZU: Studies on the Reduction of Terpenes with Sodium in Aqueous-ammonia. Part I. Synthesis of (−)-Menthol from (−)-Menthone. 376

H. UEDA and S. SHIMIZU: Studies on the Reduction of Terpenes with Sodium in Aqueous-ammonia. Part II. Synthesis of (+)-Isomenthol from Inverted Menthone. 380
N. TAKAHASHI, Y. SETA, H. KITAMURA and Y. SUMIKI: Biochemical Studies on "Bakanae" Fungus. Part 48. A New Gibberellin, Gibberellin A_4. 405
H. KITAMURA, Y. SETA, N. TAKAHASHI, A. KAWARADA and Y. SUMIKI: Biochemical Studies on "Bakanae" Fungus. Part 49. Chemical Structure of Gibberellins. Part XV. 408
Y. SETA, N. TAKAHASHI, A. KAWARADA, H. KITAMURA and Y. SUMIKI: Biochemical Studies on "Bakanae" Fungus. Part 50. Chemical Structure of Gibberellins. Part XVI. 412
S. MARUMO: Islanditoxin, a Toxic Metabolite produced by *Penicillium islandicum* Sopp. Part III. Structure of Islanditoxin. 428

Biological Chemistry

F. YOSHIDA, M. NAGASAWA and E. ICHISHIMA: Studies on the Proteolytic Enzymes of Black Aspergilli. Part V. The Specificity of Crystalline *Asp. Saitoi* Proteinase by Application of the Sanger DNP-Method. 363
I. CHIBATA and T. TOSA: Studies on Amino Acids. XII. Studies on the Enzymatic Resolution (X): Acylase Activity in Plants. 370
M. NAKAMURA: Effects of Phosphatase and Phosphoglucomutase on the Determination of Phosphorylase Activities in Crude Extracts from Plant Materials. 383
M. NAKAMURA: The Sucrose-synthesizing Enzyme in the Beans. 398

Microbiology and Fermentation Industry

H. ŌNISHI: Studies on Osmophilic Yeasts. Part V. Factors Affecting Growth of Soy Yeasts and Others in the Environment of a High Concentration of Sodium Chloride. (II). 351
H. ŌNISHI: Studies on Osmophilic Yeasts. Part VI. Glycerol Production by the Salt-tolerant Yeasts in the Medium with High Concentrations of Sodium Chloride. 359
S. NAKAMURA and T. SHIRO: Studies on Growth Inhibition of Hiochi-bacteria, Specific Saprophytes of Saké. Part IV. Hydroxyaspergillic Acid as a Growth Inhibitant against Hiochi-bacteria. 418
K. SAKAGUCHI: Growth of *Pediococcus soyae* nov. sp. in High Concentrated Solutions of Inorganic Salts and Sugars. Studies on the Activities of Bacteria in Soy Sauce Brewing. Part VI. 438
K. SAKAGUCHI: P-Factor, a New Peptidic Growth Factor for *Pediococcus soyae*. Studies on the Activities of Bacteria in Soy Sauce Brewing. Part VII. 443

Note

H. ISHIKAWA and T. OKI: Biological Conversion of Shikimic Acid or Quinic Acid to Protocatechuic Acid, Gallic Acid and Aromatic Amino Acids. 451

Short Communications

T. KISAKI, M. IHIDA and E. WADA: A New Synthesis of N-Methylmyosmine. 454

No. 6

Organic Chemistry

K. NISHIDA, A. KOBAYASHI, T. NAGAHAMA and T. NUMATA: Studies on Some New Azoxy Glycosides of *Cycas revoluta* Thunb. Part I. On Neocycasin A, β-Laminaribiosyloxyazoxymethane. 460

N. TAKAHASHI, Y. SETA, H. KITAMURA, A. KAWARADA and Y. SUMIKI: Biochemical Studies on "Bakanae" Fungus. Part 51. Chemical Structure of Gibberellins. Part XVII. 493

Y. SETA, N. TAKAHASHI, H. KITAMURA, M. TAKAI, S. TAMURA and Y. SUMIKI: Biochemical Studies on "Bakanae" Fungus. Part 52. Chemical Structure of Gibberellins. Part XVIII. 499

N. TAKAHASHI, Y. SETA, H. KITAMURA and Y. SUMIKI: Biochemical Studies on "Bakanae" Fungus. Part 53. Chemical Structure of Gibberellins. Part XIX. 509

H. UEDA and S. SHIMIZU: Studies on the Reduction of Terpenes with Sodium in Aqueous-ammonia. Part III. On the Reduction (−)-Carvone. 524

T. ITO and H. OGAWA: Chemical Studies on the Antibiotic Esperin. Part. III. On the Structure of Esperin. 536

R. SUEMITSU, M. NAKAJIMA and M. HIURA: Studies on the Metabolic Products of *Macrosporium porri* Elliott. Part III. Structure of Macrosporin (Group II). 547

Biological Chemistry

K. MUNAKATA, A. HARADA and T. NISHIZAWA: Nematocidal Activities of Halogenoalkylcarboxylic Acid Esters. 457

Y. KOBASHI, S. SAKAGUCHI and M. IZAWA: Influence of Smoking Procedures on Combustion Temperature of Cigarettes and the Nicotine Content of Cigarette Smoke. 528

Y. KOBASHI, S. SAKAGUCHI and M. IZAWA: On the Influence of Moisture Content in Cigarettes on Combustion Temperature and Transfered Amount of Nicotine into Cigarettes Smoke. 532

H. KATO: Studies on Browning Reactions between Sugars and Amino Compounds. Part IV. Identification of 5-Hydroxymethylfurfural formed from Aromatic Amine-N-Hexosides and its Role in the Browning Reaction. 551

Microbiology and Fermentation Industry

M. SATO and T. AKATSUKA: Purification of Peptidases of *Aspergillus oryzae* and Some Properties of the Purified Peptidases. 465

H. KATAGIRI and T. TOCHIKURA: Microbiological Studies of *Coli-aerogenes* Bacteria. Part VII. Degradation of Citrate. 475

H. KATAGIRI and T. TOCHIKURA: Microbiological Studies of *Coli-aerogenes* Bacteria. Part VIII. The Occurrence of Isocitritase and Citritase. 482

H. KATAGIRI and T. TOCHIKURA: Microbiological Studies of *Coli-aerogenes* Bacteria. Part IX. On the Mechanism of Assimilation of Ammonia. 489

Short Communications

Z. NAGASHIMA and M. UCHIYAMA: Possibility that Myrosinase is a Single Enzyme and Mechanism of Decomposition of Mustard Oil Glucoside by Myrosinase. 555

T. NAGAHAMA, T. NUMATA and K. NISHIDA: Neocycasin B and Macrozamin. (Studies on Some New Azoxy Glycosides of *Cycas revoluta* Thunb. Part II). 556

H. KATAGIRI and T. TOCHIKURA: The Glyoxylic Reductase-Glycollic Oxidase System in Microorganism. 558

AUTHOR INDEX TO VOLUME 23, 1959

[A]

ABE, Matazō 246
AKATSUKA, Takami 465
ANNO, Kimiko 67
ARIYAMA, Hisashi 348
ASO, Kiyoshi 342

[C]

CHIBA, Hideo 207,213,340
CHIBATA, Ichiro 370
CHIKAMOTO, Tadayoshi 171,174

[F]

FUKUMOTO, Juichiro 145,150
FUKUSHIMA, Danji 7,15

[H]

HARADA, Akio 457
HARADA, Tokuya 222
HAREYAMA, Shin'ichi 3
HASEGAWA, Takezi 137
HAYASHI, Masaki 159
HAYASHI, Ryozo 244,245
HIROSE, Yoshiyuki 140,143,253
HIURA, Makoto 547
HIZUKURI, Susumu 138
HORITSU, Hiroyuki 186
HOSHI, Reiko 67

[I]

ICHISHIMA, Eiji 363
IDA, Keiji 40
IHIDA, Masanao 454
IMAMURA, Hiroyuki 233
INOUE, Shigeharu 289
INOUYE, Yuzo 171,174
ISHIHARA, Tatsuo 178,341
ISHII, Shoziro 64
ISHIKAWA, Hisao 451
ITO, Masaharu 34
ITO, Teiichiro 289,536
IZAKI, Kazuo 310
IZAWA, Masao 194,198,201,528,532

[K]

KAJI, Akira 131
KAMEDA, Katsuzo 28
KATAGIRI, Hideo 322,475,482,489,558
KATO, Hiromichi 551
KATSUDA, Yoshio 171,174
KAWARADA, Akira 343,344,408,412,493
KISAKI, Takuro 454
KITAMURA, Hiroshi 344,405,408,412,493,499,509
KITO, Makoto 340
KOBASHI, Yūsuke 194,198,528,532
KOBAYASHI, Akira 460
KOBAYASHI, Yasuo 239
KOMATSU, Hajime 246
KONDO, Shinichi 289
KONDO, Tamio 178,233,341
KONDO, Yoichi 249
KOSHIKA, Mitsuo 107
KOYANAGI, Tatsuo 3
KŌZU, Yoshiharu 246
KUNINAKA, Akira 239,281
KUSUMOTO, Mitsugi 246

[M]

MARUMO, Shingo 428
MATSUI, Masanao 1,141,230
MIYANO, Masateru 141
MIZUTANI, Junya 121
MORI, Kazuo 79
MORI, Kenji 272,278,389
MORIHARA, Kazuyuki 49,60
MORITA, Yuhei 28
MUNAKATA, Katsura 64,457

[N]

NAGAHAMA, Tomonori 460,556
NAGASAWA, Michitaro 363
NAGASHIMA, Zenji 555
NAGATA, Ayako 89,95
NAKAJIMA, Minoru 547
NAKAMURA, Eiko 67
NAKAMURA, Kikunoshin 348
NAKAMURA, Michinori 272,278,383,389,398
NAKAMURA, Seiji 65,418
NAKAMURA, Yukihiko 79,156
NAKATSUKA, Tomoichiro 140,143,253
NAKAYAMA, Michio 46
NISHIDA, Kotaro 460,556
NISHIZAWA, Tsutomu 457

Author Index to Volume 23, 1959

NISHIZAWA, Yoshihiko ... 1
NUMATA, Tadao ... 460, 556

[O]

OBATA, Yataro ... 107, 116, 121, 186
OGAWA, Hiroshi ... 289, 536
OGAWA, Shigeru ... 64
OI, Susumu ... 150
OKADA, Shigetaka ... 145
OKI, Tae ... 451
OMURA, Hirohisa ... 83
ŌNISHI, Hiroshi ... 322, 351, 359
OTA, Minoru ... 3
OTSUKA, Shin-ichiro ... 239

[S]

SAITO, Hatsuho ... 67
SAITO, Kunio ... 89, 95
SAITO, Tetsuo ... 64
SAKAGUCHI, Kenji ... 22, 100, 438, 443
SAKAGUCHI, Kin-ichiro ... 239
SAKAGUCHI, Sōichi ... 194, 528, 532
SAKAMOTO, Kiyoshi ... 89, 95
SAKAMURA, Sadao ... 116
SASAOKA, Kei ... 347
SATO, Masakazu ... 465
SATOMURA, Yukio ... 145, 150
SAWADA, Akira ... 150
SENO, Nobuko ... 67
SETA, Yasuo ... 344, 405, 408, 412, 493, 499, 509
SHIBAHARA, Yasuo ... 376
SHICHIJI, Saburo ... 159
SHIMIZU, Sumio ... 376, 380, 524
SHIMOMURA, Tokuji ... 156
SHIRASAKA, Makoto ... 244, 245
SHIRO, Teruo ... 65, 418
SUDA, Motoshige ... 233
SUEMITSU, Rikisaku ... 547
SUGAWARA, Shiro ... 156
SUGIMOTO, Etsuro ... 207, 213, 340
SUMIKI, Yusuke ... 343, 344, 405, 408, 412, 493, 499, 509
SUZUKI, Yukio ... 322
SUZUKI, Taneko ... 68

[T]

TAKAHASHI, Heihachiro ... 83
TAKAHASHI, Kenji ... 249
TAKAHASHI, Nobutaka ... 344, 405, 408, 412, 493, 499, 509
TAKAHASHI, Takeshi ... 137
TAKAI, Makoto ... 499
TAKAKUWA, Masayòshi ... 316
TAKASAKI, Rinzi ... 245
TAKEDA, Rokuro ... 126, 165
TAKI, Motoo ... 198, 201
TAMURA, Saburo ... 499
TANAKA, Mizuho ... 68
TOCHIKURA, Tatsurokuro ... 322, 475, 482, 489, 558
TOSA, Tetsuya ... 370
TSURUTA, Masako ... 244, 245

[U]

UCHIYAMA, Masaaki ... 555
UEDA, Hiroo ... 376, 380, 524

[W]

WADA, Einosuke ... 454
WATANABE, Hiroyasu ... 257, 260, 263, 268
WATANABE, Toshiyuki ... 342
WATANABE, Wataru ... 73

[Y]

YAGISHITA, Kazuyoshi ... 217
YAMADA, Kisaku ... 68
YAMADA, Saburō ... 246
YAMAMOTO, Kisirō ... 110
YAMANO, Tōgo ... 246
YAMASHITA, Kyôhei ... 230
YAMATODANI, Saburō ... 246
YOSHIDA, Fumihiko ... 363
YOSHIDA, Shigekazu ... 34

SUBJECT INDEX TO VOLUME 23, 1959

[A]

Absidia regnieri 244
acid-fast and non acid-fast cells to lysozyme, susceptibility of 146
acid-labile phosphate compounds 277
acylase activity, in plants 370
Aerobacter aerogenes 222
A. aerogenes, reductase of 558
A. aerogenes strain A 310
(±) allethrolone-methylether 171
allyl bromoacetate, nematocidal activities of 457
D-amino acid, metabolism of 310
aminopolypeptidase, of *Aspergillus oryzae* 465
Ammonia, assimilation by *coli-aerogenes* bacteria .. 489
amperometric titration, of starch 34
α-amylase, reactivation of 68
amylodextrin 138
antibiotic 536
antibiotic kanamycin 289
aromatic amino acids, formation of 351
aspartase, of *coli-aerogenes* bacteria 489
Aspergillus oryzae, ribosidase of 281
Aspergillus sojae 7,15,100
Asp. Saitoi proteinase, specificity of 363
azoxy glucoside 556

[B]

Bacillus subtilis 100
bacteria, in soy sauce brewing 438
"bakanae" fungus 344,405,412
basic triple salt, containing magnesium hydroxide 46
beer, sunlight flavour of 186
blood pressure, elevation of 3
borer attracting component, from rice plants 64
browning reactions, between sugars and amino compounds 551

[C]

(−)-carvone, reduction of 524
α-casein 116
β-casein 116
catechin, intermediate metabolites of 257
cell membranes, permeability of 332
chloramphenicol 322,328
cholesterol levels, in the serum 3
cigarette 528,532
cigarette smoke 194,198,201,528,532
(+)-cinerolone, absolute configuration of 174
citrate, degradation by *coli-aerogenes* bacteria 475
citrate, formation of glyoxylate from 475
citrate, formation of α-ketoglutarate from 475
citrate, mechanism of degradation of 482
citritase, of *coli-aerogenes* bacteria 482
Citrus Unshiu (mandarin orange) 343
Cl. felsineum var. *sikokianum* 131
cobalt-60 73
coli-aerogenes bacteria 322
coli-aerogenes bacteria, aspartase activity of 489
coli-aerogenes bacteria, assimilation of ammonia by 489
coli-aerogenes bacteria, citritase of 482
coli-aerogenes bacteria, degradation of citrate by .. 475
coli-aerogenes bacteria, isocitritase of 482
columbin δ-lactone by 515
Corticium solani 137
Cryptomeria japonica 233
crystalline phosphoglyceric acid mutase 340
Cycas revoluta 556
Cycas revoluta Tunb, azoxy glycocides of 460

[D]

dechloro-O, O′, N-methylpyoluteorin (II), acid hydrolysis of 169
dechloro-O, O′, N-methylpyoluteorin (II), alkali fusion of 169
dechloro-O, O′, N-methylpyoluteorin (II) with chromium trioxide in acetic acid, oxidation of 170
dechloro-O, O′, N-methylpyoluteorin (II) with lithium aluminum hydride, reduction of 170
denaturation, degree of 7
2,10-dibromo-1.4-dioxo-1.4.5.8.9.10-hexahydronaphthalene 1
L-dichloroproline 428
diethyl-α-benzoyl-α-(3-(3-methylphthalidyl))-malonate 1
diethyl-α-benzoyl-α-(3-(3-methylphthalidyl))-succinate 1
dihydrogibberellin A_1 408,493,512
dihydrostreptomycin 322,323
δ-(3,4-dihydroxyphenyl)-γ-valerolactone 260
dl-δ-(3,4-dimethoxyphenyl)-γ-valerolactone 263

1,3-dimethyl fluorene 504,505
1,7-dimethyl fluorene 409,495
1,9-dimethyl fluorene 505
dipeptidase, of *Aspergillus oryzae* 465
DNP, effects on endogenous respiration of the budding cells 163
dry bakers' yeast, fermentation of 316
dry yeast 316
Duolite CS-101 131

[E]

earth metal salts, effects on bacterial α-amylase inactivated with EDTA 69
ergosecaline 246
ergosecalinine 246
Escherichia coli, gamma-ray resistivity of 73
esperin, structure of 536
2-ethylthio-1-(4-hydroxy-3-methoxyphenyl)-propanone (1) 341

[F]

free amino acid, in cigarette smoke 198,201

[G]

gallic acid, formation of 351
gamma-ray 73
gibberic acid 497
gibberellin, chemical structure of 344,408,412,493
gibberellin, relation among four isomers of 493
gibberellin A_1 343,493,499,509
gibberellins A_1, A_2, A_3 408
gibberellin A_3 (gibberellic acid) 497,500,509,513
gibberellin A_3 methyl ester, hydrogenolysis product of 510,511
gibberellin A_4 405
gibberellin A_4, isolation and characterization of 405
gibberellin A_4 methyl ester 406
gibberellin C 493,499
L-glutamic acid dehydrogenase, extracted from beef liver 249
L-glutamic acid from α-ketoglutaric acid, the formation of 249
L-glutamic acid, metabolism of 310
glycollic oxidase 558
glyoxylic reductase 558
growth factor, for *Pediococcus soyae* 443
guaiacylpropanediols, hydrogen sulfide cooking and subsequent alkali treatment of 178

[H]

halogenoalkylcarboxylic esters, nematocidal activities of 457
hiochi-bacteria, growth inhibition of 65,418
homoserine dehydrogenase 347
honey 342
humulone 188
hydroxyaspergillic acid, growth inhibitant against hiochi-bacteria 65,418
2β-hydroxylation 245
hydroxylation of steroids 244
5-hydroxymethylfurfural 551
δ-(3-hydroxyphenyl)-γ-valerolactone 260
6-hydroxypurine ribonucleosides 281
Hydroxyroteol 141
hypercholesteremia 348
hypertension 3

[I]

inorganic orthophosphate, colorimetric determination of 272
insoluble acid catalyst, for the hydrolysis 67
ion-exchange chromatography, of sugars in plant 389
irradiation, ultraviolet light 107
islanditoxin, structure of 428
isocitritase, of *coli-aerogenes* bacteria 482
isogibberellin A_1 493,512
isohibalactone 230
isoilexene 217
isoilexenedione 217
isoilexone 217
L-isoleucyl-L-valine anhydride 121
isomaltose from honey, isolation of 342
(+)-isomenthol, synthesis of 380

[J]

Japanese-radish peroxidase a 28

[K]

α-ketoglutarate, from citrate by *coli-aerogenes* bacteria 475
α-ketoglutaric acid-fermentation 322
koji 110

[L]

labelled crystalline proteinase, paperelectrophoresis of 61
α-lactalbumin 116
β-lactoglobulin 116

lactone, of gibberellins 513
laminaribiose, from reversion products of D-glucose 67
β-laminaribiosyloxyazoxymethane, see neocycasin A 460
lignin 341
lignin model compounds, hydrogen sulfide cooking and subsequent alkali treatment of 178
lipid, metabolism of 78
lupulone 189
lysozyme, action of (on acid resistant organismus) 146
lysozyme and other enzymes on the cells of acid-fast type, single or joint action of 147
lysozyme with other enzymes, joint action of (on acid resistant organismus) 147

[M]

macrosporin, structure of 547
Macrosporium porri 547
macrozamin 556
(−)-menthol, synthesis of 376
δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone 268
dl-δ-(3-methoxyphenyl)-γ-valerolactone 263
(±) methyl-1,7-dimethyl-fluorene-9-carboxylate 500
1-methyl fluorene 504,505
1-methyl-2-hydroxy fluorene 501,505
1-methyl-7-hydroxy fluorene 499,502
S-methyl methionine sulfonium 348
N-methylmyosmine, a new synthesis of 454
3-methyl-2-oxocyclopentane-1-carboxylic acid, polarography of 40
mode of fat hydrolysis and its specificities, of some fungul enzymes 153
mold maltase, crystallization of 158
mold saccharogenic amylase and maltase, fractionation of 157
monobromogibberellin A_1 494
monobromogibberellin A_3 512,513
mustard oil glucoside 555
myrosinase 555

[N]

nematocides, halogenoalkylcarboxylic esters 457
neocycasin A, structure of 460
neocycasin B 556
nicotine 528,532
nitrate reductase 83
"NS salt B", the new basic triple 46
nucleic acid by microorganisms, decomposition of 281

[O]

occidentalol 140
occidol 143,253
organic substance, determination of total phosphorus content of 278
orthophosphate, polymerization of 280
oryzanone, an attraction of the rice stem borer 64
osmophilic yeasts 332,351,359
oxalate in fish skeletal muscle, decomposition and formation of 71

[P]

paper-electrophoresis, migration distance in 89,95
Pediococcus soyae 100,443
Pediococcus soyae nov. sp. 438
Pediococcus soyae nov. sp., proteases, transaminases of 22
Penicillium citrinum 239
peptidase activity, towards glycylmethionine 371
peptidases of *Aspergillus oryzae*, effect of Zn^{++} and Co^{++} on 465
peptidases of *Aspergillus oryzae*, properties of 465
peptidases of *Aspergillus oryzae*, purification of 465
phenolic components, in cigarette smoke 194
phenolic lactones 230
phenolsulphatase, utillization of 222
D-β-phenyl-β-amino-propionic acid 428
5′-phosphodiesterase 239
phosphoglyceric acid mutase 207,213,340
phosphorylase activities, determination of 383
phyto-peroxidase 28
pine ethanol lignin 341
protease, mold 110
protease of *Pseudomonas*, factors affecting the enzyme production 49
protease of *Pseudomonas*, on the role of calcium 60
proteolytic enzymes, of black aspergilli 363
protocatechuic acid, formation of 351
pseudodihydrogibberellin A_1 494
pseudogibberellin A_1 493,512
Pseudomonas aeruginosa 126
Pseudomonas myxogenes sp., proteinase production 49
pyoluteorin 126
pyoluteorin, the structure of 165
(+) pyrethrolone, absolute configuration of 174

[Q]

quinic acid, conversion of 351

[R]

Reichstein's Compound S 137
riboflavin 3
rosenolactone, γ-lactone of 515
Roteol 141

[S]

Saccharomyces rouxii 100, 332, 351, 359
salt-tolerant yeast 332
sarkomycin, polarography of 40
Sclerotinia fungus, lipase formation of 150
Sclerotinia libertinia 245
shikimic acid, conversion of 351
smoking apparatus 529
sodium N-methyldithiocarbamate, mematocidal activities of 457
soybean flours 15
soybean flour, defatted 7
soybean proteins 7, 15
soy mash 22
soy sauce (brewing) 22, 100
starch-iodine affinity 34
Streptomyces kanamyceticus 289
sucrose-synthesizing enzyme, effect of addition of G-1-P and ATP on 401
sucrose-synthesizing enzyme, effect of divalent cations on 401
sucrose-synthesizing enzyme, in the beans··· see UDPG→fructose transglucosylase
sugars in plants, ion-exchange chromatography of 389
sugars in potatoes, effect of storage temperature on 389

[T]

terpenes, reduction of 380, 524
tetracyline 322, 329
tetracyclin analogues 1
the first budding cycle 160
Thuja occidentalis L. 140
Thuja occidentalis L., the essential oil of the wood of 253
1, 3, *p*-trimethyl fluorene 505
tyramine, utilization of 222
tyrosinase 555

[U]

UDPG→fructose transglucosylase 398
urs-13(18)-en-3β-yl acetate 217

[W]

water dispersibility, of protein 7
wood extractives 233

[Y]

yeast ribonucleic acid 239

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 1, p. 1~3, 1959]

Attempted Syntheses of Tetracyclin Analogues. Part I.

By Masanao MATSUI and Yoshihiko NISHIZAWA*

Faculty of Agriculture, University of Tokyo

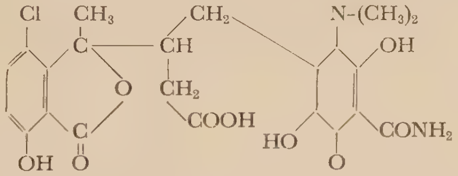
Received April 5, 1956

Diethyl-α-benzyl-α-(3-(3-methylphthalidyl))-malonate and diethyl-α-benzoyl-α-(3-(3-methylphthalidyl))-succinate as intermediates of aureomycinic acid analogues, and 2, 10-dibromo-1.4-dioxo-1.4.5.8.9.10-hexahydronaphthalene as intermediate of tetracyclin A. B ring analogue have been prepared.

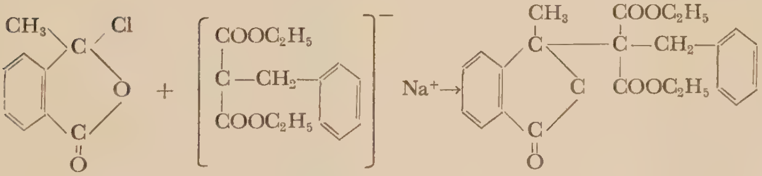
The structure which is common to both terramycin and aureomycin have been named tetracyclin by C. R. Stephens et al.[1]

We attempted to prepare the analogues of this tetracyclin and in the course a few products as the intermediates of these analogues were synthesized.

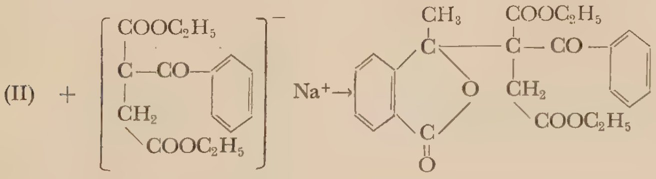
Namely, with a view of synthesizing the analogues of aureomycinic acid[2] which had been isolated as degradation product of aureomycin, the coupling reaction was first tried between 3-chloro-3-methylphthalide (acetophenon-o-carboxylic acid pseudo-acid chloride) (II) and diethyl benzoyl sodio malonate (III) and diethyl-α-benzyl-α-(3-(3-methylphthalidyl))-malonate (IV) was yielded.



(I)



(II) (III) (IV)



(V) (VI)

---

* Laboratory of Sumitomo Chem. Co., Ltd. Osaka.

1) Stephens, *J. Am. Chem. Soc.*, **74**, 4977 (1952).

2) Hutchings, *ibid.*, **74**, 4980 (1952).

This pseudo-acid chloride (II) was highly anstable and decomposed at 45°C.

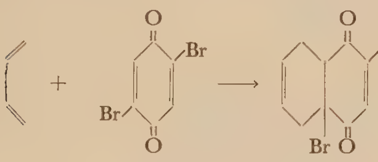
Reaction between the pseudo-acid chloride and diethyl benzoyl sodio succinate (V) yielded diethyl-α-benzoyl-α-(3-(3-methylphthalidyl))-succinate (VI).

Diethyl-α-benzoyl-α-(3-(3-methylphthalidyl))-malonate, however, was found to be quite stable to acid hydrolysis, and after twelve hours reflux in concentrated hydrochloric acid the two ester groups still retained.

Since these compounds (IV) and (VI) have two asymmetric centers, it exists in two racemic forms or diasteres isomers, but we could not isolate them because the sample was very small in quantity.

As the first attempt to prepare a tetracyclin A. B ring analogue, we tried to use Diels-Alder reaction between butadien and some benzoquinone derivatives.

The reaction between 2, 5-dimethoxy-benzoquinone and butadien did not proceed as expected and could not be obtained at all. But when 2, 5-dibromo-benzoquinone (VII) and butadien had been heated in benzene at 100°C in sealed tube for six hours, a light yellow adduct (VIII)[3] was obtained in good yield.



(VII) (VIII)

This adducts (VIII) was somewhat unstable, discoloured after a long time at room temperature, and festered skin, the solution of this adduct in benzene was specially hard and proved toxic to house flies.

One bromine-atom of this adduct was very unstable, therefore the authors could not prepared 2, 9-diacetoxy-1.4-dioxo-1.4.5.8.9.10-hexahydronaphthalen from this adduct with silver acetate.

As the studies are now in progress, they will be published in future.

EXPERIMENTAL

3-Chloro-3-methylphthalide (II)

A mixture of 8.2 g of acetophenon-o-carboxylic acid[4] and 10.4 g of phosphorus pentachloride were stirred in 30 ml. of dry benzene for two hours below 40°C. The clear solution was filtered and the filtrate was evaporated *in vacuo* at room temperature and petroleum ether (50 ml.) was added. After cooling for one day the solid was filtered and washed with petroleum ether; yield 8.0 g. This product was not further purified but was used for the further reactions.

Diethyl-α-benzyl-α-(3-(3-methylphthalidyl))-malonate (V)

To a stirred dry benzene solution suspended with 0.9 g of sodium-sand was added 10 g of diethyl-α-benzyl-malonated[5] dissolved in the equal quantity of dry benzene. The mixture was refluxed for four hours.

After the sodium-sand was dissolved completely a suspension of 3 g of 3-chloro-3-methylphthalide in 20 ml. of dry benzene was added to the solution at room temperature.

Stirring was continued for one hour at room temperature then the mixture was refluxed until it became neutral, cooled, and the insolubles were removed by filteration. The clear benzene solution was concentrated to dryness *in vacuo* and the dark red residual oil was diluted with alcohol.

The crystalline product was filtered and dried, wt. 4.0 g, m.p. 141–143°. A portion of it was crystallized twice from acetic acid; m.p. 145–146°. *Anal.* Calcd. for $C_{23}H_{24}O_6$; C, 69.68; H, 6.10

Found: C, 69.33; H, 6.25

Diethyl-α-benzoyl-α-(3-(3-methylphthalidyl))-succinate (VI)

This was prepared using 0.5 g of sodium-sand, 6.1 g of α-benzoyl-succinate[6] and 4.1 g of 3-chloro-3-methylphthalide by the above discribed methods.

The crystalline product was 3.2 g, m.p. 220–221°C. *Anal.* Calcd. for $C_{23}H_{24}O_7$; C, 67.91; H, 5.70

Found: C, 67.88; H, 5.76

2.10-Dibromo-1.4-dioxo-1.4.5.8.9.10-hexahydronaphthalene (VIII)

Five and four tenth grams of 2.5-dibromo-*p*-benzoquinone[7] (m.p. 188°), 1.6 g of freshly distilled butadien; 40 ml. of benzene were shaken in seald tube at 100°C for six hours. The light-yellow reaction mixture was filtered, and concentrated to dryness *in vacuo*.

---

[3] Matsui and Nishizawa, Jap. pat. No. 227,481.

[4] Gabriel, *Ber.*, **10**, 1553.

[5] Leuchs, *Ber.*, **44**, 1509.

[6] Perkin, *J. Am. Chem. Soc.*, **47**, 273 (1925).

[7] Sarauw, *Ann.*, **209**, 112 (1881).

The crude product was recrystallized from alcohol; 5.3 g, m.p. 94–95° *Anal.* Calcd. for $C_{10}H_8O_2Br_2$; C, 37.50; H, 2.5

Found: C, 37.38; H, 2.31

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 1, p. 3~6, 1959]

The Effect of Riboflavin on Hypertension induced by High Salt Diet in the Rat

By Tatsuo KOYANAGI, Shin'ichi HAREYAMA and Minoru OTA

Department of Agricultural Chemistry, Faculty of Agriculture, Iwate University

Received November 19, 1956

The effect of riboflavin on development of hypertension in rats given a high salt diet was studied. Large doses of riboflavin prevented both elevation of blood pressure and rise of cholesterol levels in the serum. The increase in liver monoamine oxidase activity of the rats fed riboflavin was confirmed.

Calder[1] reported that rats fed a vitamin B-deficient diet showed a higher level of blood pressure than those fed a similar diet supplemented with yeast or liver extracts and considered that the vitamin B group contained in the supplements may inhibit the elevation of blood pressure of the animals. The present study was designed to determine whether riboflavin is effective to inhibit the elevation of blood pressure in rats induced by a salt-rich diet.

EXPERIMENTAL AND RESULTS

The first experiment; A preliminary examination was performed to find the effect of riboflavin on salt-hypertension occurring in animals. In this study, ten two-month-old Wister strain albino rats were divided into two groups; one of them was fed a basal diet consisting of 91.8 g polished rice, 5 g casein, 0.5 g calcium carbonate, 2.5 g sodium chloride, 0.2 g dried carrot powder and 0.1 mg thiaminehydrochloride, the other was fed a basal diet plus 1 mg riboflavin. The basal diet contained 0.071 mg riboflavin. The diet which was pasted by heating with 100 ml water per 100 g was given ad. lib. and drinking water was restricted according to the suggestion of Sapirstein[2] that the ratio of sodium chloride to water intake, rather than the amount of sodium chloride, is considered to be the determining factor for development of this type of hypertension.

After three months' feeding, systolic blood pressure of the rat was measured by the method of Williams et al.[3] and then, the animals were sacrificed by decapitation and their liver and kidney removed immediately so as to serve for the determinations of

---

[1] R. M. Calder, *J. Exp. Med.*, **76**, 1 (1942); **79**, 215 (1944)

[2] L. A. Sapirstein, W. L. Brandt, and D. R. Drury, *Proc. Soc Exp. Biol. Med.*, **73**, 82 (1950).

[3] J. R. Williams, T. R. Harison and A. Gollman, *J. Clin. Invest.*, **18**, 373 (1939).

TABLE I. BLOOD PRESSURE AND ENZYME ACTIVITIES IN LIVER AND KIDNEY OF RATS ON HIGH SALT DIETS WITH AND WITHOUT RIBOFLAVIN

Group	Body Weight	Sex	Blood Pressure	Activity of Liver	Succino Oxidase Kidney	Activity of Liver Monoamine Oxidase
Control Group	248 g	F	125 mm Hg	46.2 μl*	42.9 μl*	20.3 μl*
	250	M	105	35.4	38.9	21.1
	190	F	132	25.9	31.3	17.5
	175	F	122	65.7	69.3	17.3
	189	F	97	64.4	89.2	18.4
	204	F	120	62.6	72.8	17.9
Experimental Group	226	F	97	45.5	56.1	22.0
	226	M	92	81.5	79.7	31.1
	213	F	95	58.9	91.4	21.4
	174	F	97	67.7	66.7	28.2

* Values express O_2 μl consumed per 10 min. per 20 mg of fresh liver in succino oxidase and per 100 mg of fresh liver in monoamine oxidase.

enzyme activities. The activities of succino oxidase and monoamine oxidase were determined by the method of Schneider and Potter[4] and Blashko[5], respectively.

The results are presented in Table I. In this preliminary experiment, it was shown that an elevation of blood pressure in animals fed on a salt-rich diet is prevented by the addition of riboflavin to the diet, and further that riboflavin increases the activity of liver monoamine oxidase. Moreover, a hypertrophy of the spleen was observed in the riboflavin deficient rats.

The second experiment; As the influence of riboflavin on the elevation of blood pressure was recognized in the preliminary experiment, a similar study in which additional groups were fed on 1 per cent and 5 per cent salt diets was repeated. Six-week-old rats of the Wister strain were divided into six groups and fed on diets as shown in Table II. Besides, three drops of shark liver oil were given to each animal every fourth-day. As a rule, no drinking water was supplied. However, as the animals fed 5 per cent salt diet lost their appetite and stopped the increase in body weight was not observed after five weeks' feeding, they were allowed to take drinking water for the following two weeks. Thereafter, drinking water was supplied on several occasions when serious weight loss occurred.

At the 18th weeth, blood pressure was measured in the same way as described above, and then the rats were sacrificed. Immediately after decapitation, liver monoamine oxidase was determined. In the 2.5 per cent salt group, the riboflavin content of the liver of the rats was estimated by Fujita's lumiflavinfluorometry[6] and cholesterol concentration of the blood expelled from the cervical vessels at the decapitation was estimated according to the method of Sperry and Webb[7].

The results are shown in Tables III and IV. Although no influence of riboflavin on the blood pressure was observed in the 1 per cent salt diet group, a significant inhibiting action of the vitamin on the elevation of blood pressure was recognized in both high salt diet groups. As presented in Table III, the ac-

TABLE II. COMPOSITION OF DIET OF THE SECOND EXPERIMENT

No. of Group	Polished Rice	Casein	Soybean Cake	Calcium Carbonate	Sodium Chloride	Thiamine	Riboflavin
I	88.5 g	5 g	5 g	0.5 g	1 g	0.1 mg	0 mg
II	88.5	5	5	0.5	1	0.1	1
III	87.0	5	5	0.5	2.5	0.1	0
IV	87.0	5	5	0.5	2.5	0.1	1
V	84.5	5	5	0.5	5	0.1	0
VI	84.5	5	5	0.5	5	0.1	1

---

[4] W. C. Schneider and V. R. Potter, *J. Biol. Chem.*, **187**, 97 (1950).

[5] H. Blashko, D. Richter and H. Schlossman, *J. Physiol.*, **90**, 1 (1939).

[6] A. Fujita, *Vitamin*, **2**, 254 (1950).

[7] W. M. Sperry and M. Webb, *J. Biol. Chem.*, **187**, 107 (1950).

TABLE III. BLOOD PRESSURE AND LIVER MONO-AMINE OXIDASE ACTIVITIES OF THE EXPERIMENTAL ANIMALS

Group	Diet	Body Weight of Rat	Sex	Blood Pressure	Liver amine Oxidase
I	1 per cent NaCl	234 g	M	— mm Hg	34 μl*
		235	M	85	20
		210	F	90	33
		264	M	100	14
		320	M	90	25
Average		262		91	25.2
II	1 per cent NaCl Riboflavin	318	M	90	41
		260	M	80	29
		236	F	90	23
Average		270		87	31
III	2.5 per cent NaCl	230	F	100	28
		252	F	105	20
		163	F	90	—
		200	F	95	24
		238	F	100	24
Average		216		98	24
V	2.5 per cent NaCl Riboflavin	177	F	95	25
		310	M	85	48
		302	M	90	28
		325	M	80	15
		343	M	85	24
Average		291		89	27.4
V	5 per cent NaCl	235	M	100	29
		174	M	110	26
		127	M	105	—
		—	F	105	—
Average		179		105	27.5
VI	5 per cent NaCl Riboflavin	283	M	95	34
		212	F	95	27
		313	M	90	—
		210	F	100	34
Average		255		95	32

	F-Test	Liver Amine Oxidase
F-Value (5 per cent Point)	Between I and II	2.22
	Between III and IV	1.21
	Between V and VI	5.52

* Value represent O_2 μl consumed per 10 min., per 100 mg of liver.

TABLE IV. AMOUNT OF LIVER RIBOFLAVIN AND SERUMCHOLESTEROL LEVELS OF EXPERIMENTAL ANIMALS

Diet	Liver Riboflavin	Serum Cholesterol
2.5 per cent NaCl	2.21 mg %	65.7 mg %
	1.79	46.8
	1.85	65.2
	2.00	60.0
	1.73	63.1
Average	1.91	60.2
2.5 per cent NaCl Riboflavin	2.27	32.8
	2.29	28.6
	3.44	57.6
	3.20	—
	3.05	54.2
Average	2.95	43.3

tivities of liver monoamine oxidase of the animals given riboflavin in both low and high salt diets, were slightly higher than animals fed on the riboflavin deficient diet the F value ranging from 1.21 to 5.52. Changes in tissue of the liver and kidney were observed and fat degeneration of the liver was also noticed in the animals fed on the 5 per cent salt diet.

DISCUSSION

Sapirstein²⁾ and Meneely⁸⁾ have demonstrated that a high salt diet is an important factor to induce hypertension. Dahl⁹⁾ has proposed the hypothesis that men fed on a comparative high salt diet tend to be subject to hypertension. The authors in their present study observed that the animals fed salt rich diets develop an arterial hypertension and that the addition of riboflavin to the diet prevents elevation of blood pressure. Linder¹⁰⁾ has reported that the excretion of sodium chloride in urine is increased by the injection of riboflavin in human subjects. Therefore, one possibility for the prevention of hypertension by riboflavin treatment is that a large dose of this vitamin increases the excretion of urinary sodium chloride and at the same time inhibits its increase in extracellular fluid.

Hawkins¹¹⁾, and Koyanagi and Ota¹²⁾ have reported that the monoamine oxidase activity of rat liver in flavin-deficiency is decreased.

---

8) G. R. Meneely, R. G. Tucker, W. J. Darby and S. H. Auerbach, *J. Exp. Med.*, **98**, 571 (1953).

9) L. K. Dahl and R. A. Love, *Arch. Int. Med.*, **94**, 525 (1954).

10) L. H. Linder, *Wiener Klin. Woch.*, **8**, 918 (1940).

11) J. Hawkins, *Biochem. J.*, **51**, 399 (1952).

12) T. Koyanagi and M. Ota, *J. Faculty Agri. Iwate Univ.*, **2**, 175 (1955).

The authors have observed a similar result in the preliminary experiment of the study though in the second experiment, the decrease was somewhat small. Monoamine oxidase is concerned with the detoxication of the pressor amine formed in the intestine and the kidney and therefore, a high activity of the oxidase is considered to be one of the various factors causing inhibition of the elevation of blood pressure.

The present authors have found that riboflavin reduces serum cholesterol levels of rats fed a high salt diet, significantly. It is well known that the high cholesterol concentration in serum is observed more frequently in hypertensive patients as compared with normotensive subjects. However, contrary to the author's result, Myasnikov[13)] observed the inability of riboflavin to decrease the serum cholesterol levels of rabbits. This discrepancy seems to result from the difference between the above author's diet and the high salt diet used in the present experiment.

Thus, riboflavin seems to have three different actions in respect of preventing the occurrence of hypertension caused by a high salt diet, namely, the first, in increasing of the excretion of sodium chloride, the second, in strengthening amine detoxication ability and the third, in inhibiting the increase of blood cholesterol.

SUMMARY

The effect of riboflavin on hypertenstion induced by high salt diets in rats was studied and the following results were obtained.

1. Riboflavin was found effective to inhibit the elevation of blood pressure in rats fed on high salt diets.
2. Riboflavin slightly increased the activity of liver monoamine oxidase.
3. Riboflavin reduced serum cholesterol levels, significantly in rats fed on high salt diets.

---

13) A. L. Myasnikov, *Chem. Abst.*, **44**, 9024 (1950).

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 1, p. 7~14, 1959]

Studies on Soybean Proteins

Part I. Water Dispersibility of Protein of Defatted Soybean Flour as a Criterion for Degree of Denaturation*

By Danji FUKUSHIMA

Noda Institute for Scientific Research, Noda-Shi, Chiba-Ken, Japan

Received April 1, 1958

Concerning the heat treatment of defatted soybean flour, the fitness of water dispersibility of protein as a criterion of the degree of denaturation has been investigated. Denaturation was measured by the change of susceptibility of the protein in water extract of the flour to protease action of *Aspergillus sojae* before and after heat treatment. It was found that the decrease of water dispersibility did not always run paralell with denaturation. The dispersibility method was applicable only to flours autoclaved for a definite period which varied according to temperature and the water content of the flour treated.

Discussion is made about the fitness of measuring the degree of denaturation by protease action.

INTRODUCTION

Shoyū (Japanese soy sauce) and miso are foods of daily necessity to the Japanese just like butter and cheese in Western countries. They have occupied a very important position in the food industries in this country. In particular, shoyū has been mass-produced on a large scale in modern industry.

Soybean and wheat are used as materials. They are autoclaved, inoculated with seed-koji (*Aspergilli*) and incubated for four days so as to grow the mold. The resulted mold koji is added with saline and incubated for approximately one year at room temperature. The soybean protein occupies about 70% of the protein in materials. The major component of protein in soybean is "glycinin". This protein is chiefly hydrolyzed by the proteases of *Aspergilli* during brewing time to give a good taste and flavour, and high nutritive value. Therefore in shoyū production it is essential to decompose the protein in materials easily and completely by the proteases in koji-mold. Since glycinin is a globular protein and the globular proteins are, in general, hardly hydrolyzed in the native state, the soybean protein must be first denatured by some procedure. Hence the denaturation of soybean proteins is a most fundamental and important subject to be investigated.

Studies on the denaturation of soybean protein have been chiefly performed on the denaturation during the oil-extracting process. With regard to its criterion the measurement of water- or salt-dispersible nitrogen has been performed. Particularly, water dispersibility has been regarded as the best criterion1) for determining the degree of denaturation, because native soybean meal has a high dispersibility of nitrogenous constituents by water. This dispersibility is sensitively decreased by heat or practically by any other denaturing agent. The method of the water extraction has been investigated in detail by a number of workers.2,3,4)

---

* Presented at meeting of the Aricultural Chemical Society of Japan, Tokyo, September 28, 1957.

1) S. J. Circle, "Soybeans and Soybean Products, Vol. 1", Interscience Publishers, Inc., New York, 1950, p. 275.

2) A. K. Smith, S. J. Circle and G. H. Brother, *J. Am. Chem Soc.*, **60**, 1316 (1938).

3) A. K. Smith and S. J. Circle, *Ind. Eng. Chem.*, **30**, 1414 (1938).

4) R. H. Nagel, H. C. Beckel and R. T. Milner, *Cereal Chem.* **15**, 463 (1938)

It is an unusal characteristic that a very high percentage of nitrogenous matter (more than 90%) can be extracted with water from soybean meal, which differentiates soybean meal protein from other seed meal proteins. Several explanations on the high water dispersibility of soybean protein have been offered such as the potassium phosphate theory (Osborn and Campbell; 1898 and Harvath; 1937), dialyzable salt theory (Ryndin; 1936), phosphatide theory (Nagel, Beckel, and Milner; 1938), and alkalinity theory (Smith, Schubert, and Belter; 1955). However, any satisfactory explanations have not yet been obtained.

In the meanwhile, it is generally known that the degree of coagulation of proteins is greatly influenced by various factors of environment, regardless of its denaturation state. Denaturation appears to be a quite different process occurring at the different rate from that of coagulation.[5,6] Accordingly, it must be fully investigated whether or not the degree of denaturation runs paralell with the decrease of water dispersibility of soybean meal protein. However any investigation on this problem has not yet been found in the literatures. In this paper the native protein in the water extract was measured by the change of the susceptibility to the protease action of *Asp. sojae* and the fitness of water dispersibility as a criterion of the degree of denaturation was investigated.

MATERIAL AND METHODS

Preparation of Oil-Free Soybean Flour. Shirahana soybean, the variety of late-ripening, grown in 1956 in Japan were used throughout this experimental work. Material was stored in the refrigerator after it was sun-dried. Fat-free soybean was prepared by dehulling, grinding followed by the screening through a 30-mesh, then removing the oil with *n*-hexan extraction at 35°C and air-dried. The fat-free flour had been kept in refrigerator less than two months to avoid denaturation during storage. A typical fat-extracted flour sample showed the following percentage composition: moisture, 6.59; nitrogen, 8.559.

Heat Treatment of Soybean Protein. One gram of defatted soybean flour and a definite amount of water were mixed in a 25-ml Erlenmeyer flask and autoclaved.

Determination of Water-Dispersible Nitrogen. The single extraction method of Smith et al.[3] was modified as follows. Heat-treated soybean flour was transferred to a 500-ml polyethylene bottle and shaken mechanically for sixty minutes at 30°C with 200 ml water. The contents of the bottle were transferred to a 250-ml graduated flask, and the volume was adjusted to 250 ml by water. A portion was centrifuged at 2440×g for six minutes. Total nitrogen was determined on a 20 ml-sample, the supernatant from centrifugation, by the semi-micro Kjeldahl method.

Enzyme Preparation.

(1) Protease of *Asp. sojae*: The alkaline protease was isolated from *Asp. sojae* by the method of Mizunuma et al.[7]; the method included purification of the enzyme by ion exchange chromatography (IRC-50) of the water extract of a wheat bran koji. The resulted preparation contained less than 1% of original amylase activity and 2% of neutral protease, but no acidic protease.

(2) The crystalline protease of *Bac. subtilis* (Purchased from Nagase Co., Ltd.):

Determination of Protease Activity. Casein digesting activity was measured by a modification[8] of the Anson method[9]. One ml of 2% milk casein (Hammarsten) solution was poured into a test tube containing 0.5 ml of the enzyme solution and 0.5 ml of 0.2 mol. phosphate buffer (pH 7.2) and the mixture was incubated at 30°C for ten minutes. Then 2 ml of 0.4 mol. trichloroacetic acid was added to the digest to stop the reaction and the resulted precipitate was removed by filtration after standing for twenty minutes at 30°C. Five ml of 0.4 mol. sodium carbonate and 1 ml of Folin's reagent[10] were successively added to 1 ml of the filtrate and it was kept at 35°C for twenty minutes. Optical density was measured using a EPO-B Type Hitachi Photometer with a 660 mμ filter against distilled water. The same experiment was carried out with a blank which was operated essentially in the same way as above except that the enzyme was added later. Optical density of the blank test was subtracted from that of

---

[5] S. Mizushima and S. Akabori, "Chemistry of Proteins, Vol. 2" Kyoritsu Press, Japan, 1954, p. 549.

[6] K. J. Laidler, "Introduction to the chemistry of Enzymes", McGraw-Hill Book Company, Inc., 1954, p. 145.

[7] T. Mizunuma and N. Iguchi, This Bulletin, **22**, 35 (1958)

[8] B. Hagihara, *Ann. Rep. Soc. Osaka Univ.*, **2**, 35 (1954).

[9] M. L. Anson, *J. Gen. Physiol.*, **22**, 79 (1938).

[10] O. Folin and V. Ciocalteu, *J. Biol. Chem.*, **73**, 627 (1927).

the test run and converted into mg equivalent tyrosine liberated by enzyme action. One unit, $[\mathrm{PU}]^{\mathrm{cas.\,30°FR}}_{\mathrm{m.\,eq.\,tyr.}}$, represented the activity which liberated 1 mg equivalent of tyrosine in trichloroacetic acid-soluble fraction per minute under the above conditions.

Enzymatic Hydrolysis of Water-Extracted Soybean Protein. A mixture of 1 ml of substrate solution, 1 ml of enzyme solution, and 0.5 ml of 0.2 mol. phosphate buffer solution (pH 7.2) was incubated at 30°C for a definite time. One ml of 1.2 mol. trichloroacetic acid was added to stop the reaction and the filtration was done after complete formation of the precipitate. One ml of the filtrate was assayed for protein as described above. The value was expressed as the correspondent amount of tyrosine (γ) which represented the degree of hydrolysis.

RESULTS

I. Water Dispersibility of Soybean Flour Protein

(1) Effect of Time and Temperature an Autoclaving: To examine whether water dispersibility is adequate as a criterion of the degree of denaturation of soybean flour protein, the effect of time and temperature in autoclaving wasinvestigated. Fig. 1 shows the effect of time and temperature in autoclaving on water dispersibility with soybean flour to which an equal amount of water was added. At 100°C, the extracted nitrogen gradually decreased up to one hundred and twenty minutes, when dispersibility reached its minimum. At 110°C or 120°C, minimum dispersibility occurred after a few minutes and afterwards extracted nitrogen increased. The higher the temperature, the larger was the increase of the dispersed nitrogen. These results showed the unfitness of water dispersibility as a criterion of denaturation because water dispersibility of the soybean protein increased with heating time after its minimum point was attained.

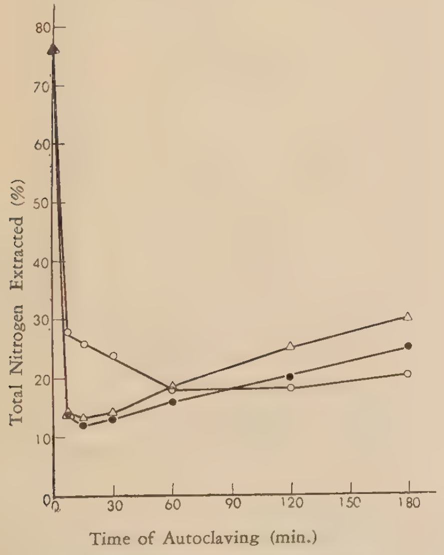


FIG. 1. Effect of Time and Temperature on Water Dispersibility of Soybean Flour Protein Autoclaved under Addition of 100% Water.

—○— 100°C, —●— 110°C, —△— 120°C

(2) Effect of Water Content: The water content of flour before autoclaving had great influence on water dispersibility as shown in Figs. 2 and 3. Fig. 2 shows the water-dispersible nitrogen of the flour autoclaved at 120°C with and without the addition of an equal amount of water. The amount of water-dispersed nitrogen was larger in the flour added with water than in flour without water addition. In fact, denaturation occurred more easily in the former than in the latter, as described later.

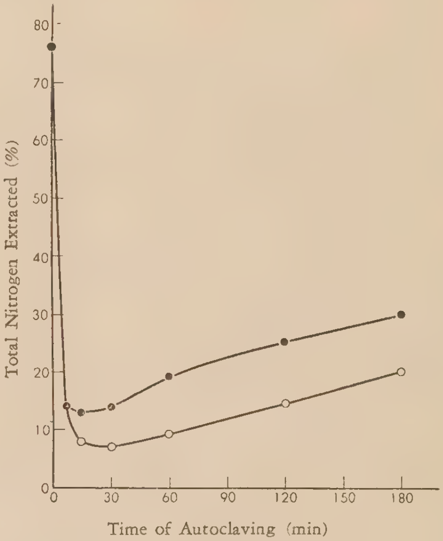


FIG. 2. Effect of Time and Water Content on Water Dispersibility of Soybean Flour Protein Autoclaved at 120°C.

—○— Without addition of water
—●— With addition of water

After autoclaving the samples containing different levels of water at a definite temperature (100°C) for

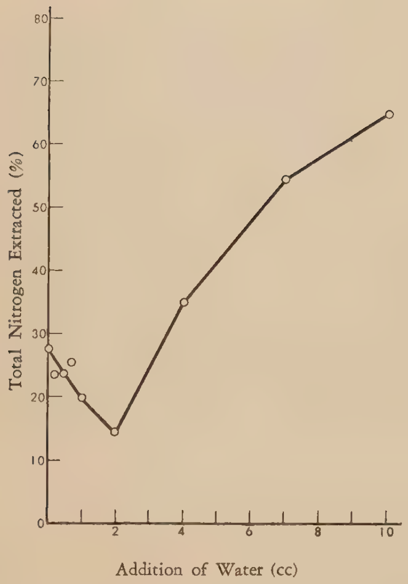


FIG. 3. Effect of Water Content on Water Dispersibility of Soybean Flour Protein Autoclaved at 100°C for Thirty Minutes.

definite period (thirty minutes), the minimum of water-dispersed nitrogen was observed by the addition of 200% water as shown in Fig. 3.

II. Determination of Native protein in Soybean Water Extract

From the above results, it was recognized that water dispersibility is not a good criterion of denaturation, because denatured soybean flour protein was extracted without coagulation under a certain condition. On account of this the measurement of the native protein in water extract by protease action was examined.

The water extract of native flour was divided into two parts: the one was added with the proteases without heating the flour and the other was heated at 100°C for ten minutes before protease addition. The results are shown in Figs. 4 and 5. As shown the protein of the heated aqueous extract was digested much faster than that of the unheated one. In this case no precipitates were produced in the water extract of flour, as long as it was diluted below definite concentrations. Moreover, there was no substance inhibitory to protease action of *Aspergillus sojae* or *Bacillus subtilis* in the extract (Table I). Accordingly, it was reasonable to attribute this increased susceptibility of protein in the heated water extract to protease action to modification of the native protein itself. Thus, the following techniques were set up for determining the native protein in solution. If necessary, the solution was diluted, and then it was divided into two parts, one being unheated and the other heated at 100°C for ten minutes.

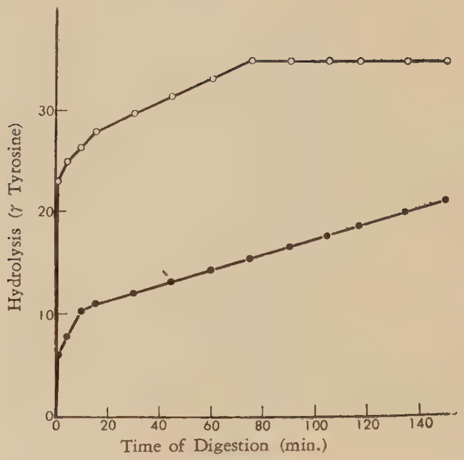


FIG. 4. Course of Hydrolysis of Soybean Water Extract by *Asp. Sojae* Protease.

—○— Treated at 100°C for fifteen min.
—●— Not treated
Total nitrogen of substrate: 0.48 mg/cc

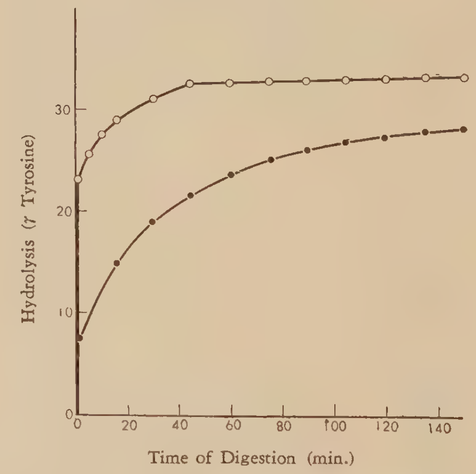


FIG. 5. Course of Hydrolysis of Soybean Water Extract by *Bac. Subtilis* Protease.

—○— Treated at 100°C for fifteen min.
—●— Not treated
Total mitrogen of substrate: 0.48 mg/cc

TABLE I. EFFECT OF SOYBEAN WATER EXTRACT ON PROTEASE ACTION

Method: Experimental conditions were the same as "Determination of Protease Activity" in the present paper. The value was expressed in γ tyrosine.

	Protease of *Asp. Sojae*		Protease of *Bac. Subtiles*
	Refined Alkaline Protease	Water Extract of Wheat Bran Koji	Crystalline Protease
Control	24.8	23.8	33.1
(Control)+(Soybean Water Extract)	23.1	23.0	32.0

Each part was added simultaneously with the alkaline protease of *Asp. sojae* (total activity; $10[\mathrm{PU}]^{\mathrm{cas.\ 30°FR}}_{\mathrm{m.\ eq.\ tyr.}}$), and incubated for thirty minutes under the same conditions described in "Materials and Methods". The difference of value between the two parts represented the amount of native protein in solution. If measurement was made in samples of the definite concentrations of protein, this difference was proportional to the amount of the native protein in the sample as shown in Fig. 6. Figs. 7, 8, 9 shows the amount of native protein* in water extracts of soybean flours autoclaved under various conditions, by the application of this method.

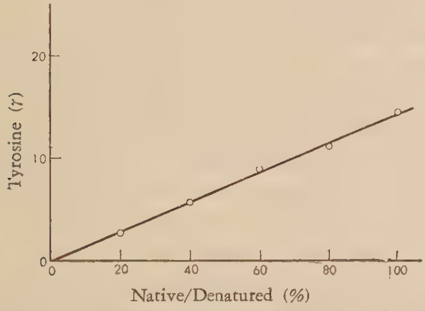


FIG. 6. Relationship between Amount of Native Protein in Solution and Value Obtained by this Method.

Substrate: Total nitrogen 0.4 mg/cc. The ratio of the unheated water extract to the heated (100°C, 10 min) water extract was varied as indicated.

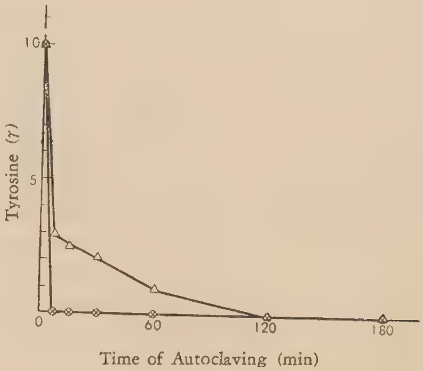


FIG. 7. Native Protein in Water Extract of Soybean Flour Autoclaved under Addition of 100% Water.

—△— 100°C —○— 110°C —×— 120°C

By autoclaving at 100°C under the addition of 100% water to the flour, the amount of native protein in the water extract gradually decreased with heating time and reached zero after one hundred and twenty minutes, whereas at 110°C and 120°C, the native protein in the water extract disappeared after heating for several minutes (Fig. 7). The amount of native protein in the water extract always reached zero at the minimum of water-dispersible nitrogen (Fig. 1, Fig. 7). The longer the heating time beyond the minimum was, the more total dispersed nitrogen amounted. A part of this increased-nitrogen was originated in denatured protein.

It was very important to examine the effect of water content on denaturation and water dispersibility of proteins. The flour autoclaved under the addition of 100% water was larger in water-dispersed nitrogen than that under no addition. On the other hand, the

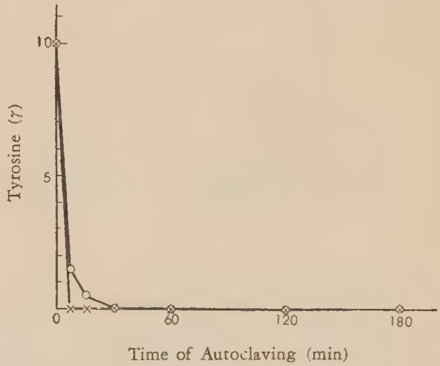


FIG. 8. Native Protein in Water Extract of Soybean Flour Autoclaved at 120°C.

—○— Without addition of water
—×— With addition of 100% water

---

* Strictly this value is not quantitative, because the protein concentration of the substrates was not regulated.

native protein in water extract assayed by the enzymatic method already reached zero after heating for a few minutes in flour containing 100% water, while it required more than thirty minute's heating to be completely denaturated in the flour heat-treated without water addition (Fig. 8, Fig. 2). In Fig. 2 and Fig. 8 as well as in Fig. 1 and Fig. 7, the time when native protein disappeared coincided with the point at which the dispersed nitrogen reached its ninimum. Fig. 9 showes the effect of water addition to flour prior to autoclaving on the amount of native protein contained in the water extract. By autoclaving the flour contained twice as much water at 100°C for thirty minutes, the resulted water extract contained no native protein; the minimum of the water-dispersed nitrogen occurred under the same conditions. In spite of the abrupt increase of water-extracted nitrogen beyond the minimum point, native protein never existed in the extract. Also, in this case the native protein in water extract completely disappeared when water-dispersed nitrogen reached its minimum. It therefore appears that the higher the water content became, the more easily the flour protein is denatured.

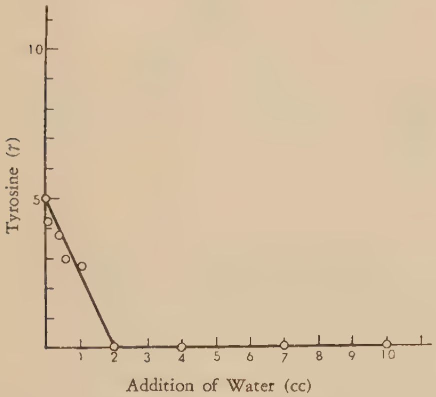


FIG. 9. Native Protein in Water Extract of Soybean Flour Autoclaved at 100°C for Thirty Minutes.

DISCUSSION

The possibility of using protease for measurement of the degree of denaturation has been suggested by several workers. Linderstrøm-Lang et al.[11] presented data indicating that native lactoglobulin might not be entirely attacked by enzyme and Haurowitz et al.[12] have pointed out that the susceptibility of native fibrous proteins to the action of trypsin is attributed to the expanded configulation of their peptide chains rendering their peptide bonds accessible to the enzyme. Lineweaver et at.[13] and Rice et al.[14] tried to measure the degree of denaturation of human serum albumin or hemoglobin by means of papain susceptibility. Bernheim et al.[15] carried out measurements of the rates of tryptic hydrolysis of horse serum albumin and horse pseudoglobulin in an attempt to detect differences in the internal structure of some proteins, i.e., native, irreversibly denatured, and the so-called regenerated proteins. Recently, Okunuki et al.[16,17] have described a quantitative method for the determination of the degree of denaturation of enzyme proteins, using crystalline protease of *Bac. subtilis* which did not act on the native protein of enzymes and hydrolyzed the denatured enzyme protein quantitatively. These authors found that the inactivation of crystalline enzymes, such as Taka-amylase and *Bac. subtilis* amylase, is proportional to the degree of hydrolysis by the protease.

Globular proteins, in general, increase susceptibility to the protease after denaturation. This has been explained from various observations[18] as follows. The atomic groups serving as points of combination with active centers of the protease are located inside the molecule in native globular proteins and are inaccessible to the protease. Denaturation which apparently involves an unfolding of the closely packed peptide chains, renders these groups accessible to the protease. Therefore, the value of the increase of susceptibility of the protein to the protease should be considered to be that directly reflecting the expanded configulation of the molecule. The major proteins of soybean are of the globular type and increase susceptibility to protease as shown in Figs. 4 and 5. Therefore, the measurement of the native protein in soybean meals by using protease should be regarded to be the most suitable method.

---

[11] K. Linderstrøm-Lang, R. D. Hotchkiss and G. Johansen, *Nature*, **142**, 966 (1938).

[12] F. Haurowitz, M. Tunca, P. Schwerin and V. Goksn, *J. Biol. Chem.*, **157**, 621 (1945).

[13] H. Lineweaver and S. R. Hoover, *J. Biol. Chem.*, **137**, 325 (1941).

[14] R. G. Rice, G. A. Ballou, P. D. Boyer, J. M. Luck and F. G. Lumi, *J. Biol. Chem.*, **158**, 609 (1946).

[15] F. Bernheim, H. Neurath, and J. Q. Erickson, *J. Biol Chem.*, **144**, 259 (1942).

[16] K. Okunuki, B. Hagihara, H. Matsubara, T. Nakayama and M. Yanagida, *Symposia on Enzyme Chemistry (Japan)*, **9**, 1 (1954).

[17] K. Okunuki, B. Hagihara, H. Matsubara and T. Nakayama, *J. Biochem. (Japan)*, **43**, 453 (1956).

[18] F. Haurowitz, "Chemistry and Biology of Proteins", Academic Press, New York, 1950, p. 127.

The proteases suitable for the measurement of native protein in water extract of soybean meal or flour should have its optimum pH around the neutral pH and not be inhibited by any constituents in the water extract. It was found that both protease of *Aspergillus sojae* and *Bacillus subtilis* are suitable for the above conditions (Fig. 3, Fig. 4). Nevertheless, protease of *Aspergillus sojae* was used in this paper because protease of *Aspergillus sojae* had less activity toward native protein than that of *Bacillus subtilis* though both proteases hydrolyzed it gradually.

Inadequacy of water dispersibility as criterion of the degree of denaturation is shown in this paper. However, this method serves as a criterion for the flours autoclaved within the range where the minimum point is not reached, because in this range the amounts of the native protein in water extract run paralell to water dispersibility. It should be mentioned, however, that even within the above range the absolute amount of nitrogen dispersed by water after heat treatment varies significantly depending upon the temperature or water contents of flour. And, with regard to the samples which are treated at different conditions of temperature and water content of flour, the dispersed nitrogen does not always run paralell to the amount of the native protein. For this reason, the dispersibility method is not satisfactory for comparison of the degree of denaturation of these samples. That the denatured soybean protein is solubilized by water content, may be explained in connection to the fact[19] that the coagulation of denatured proteins is inhibited by the existance of soluble protein.

Hitherto studies for denaturation of soybean proteins have been carried out in regard to heat denaturation during oil extraction and the water dispersibility method has been used as the only useful criterion for the degree of denaturation of soybean meal protein. Further, so far, the water dispersibility method has not been found to be inadequate. This is due to the following reason. During the oil-extracting process the treatment of samples has been carried out at a lower temperature and with a lower water content of flour. On account of this the measurement of denaturation resulted has only been performed upon samples which have not attained a minimum point of water dispersibility. Thus, water dispersibility method has been used as a criterion of denaturation without considerable contradiction being recognised.

Since the protein in native soybean flour is very soluble in water, the native protein in heat-treated flour is expected to be solubilized easily and completely by water. On account of this, the protease method described here is usuable for measurement of the total amount of the native protein contained in soybean flour itself. Whether or not the native protein in heated flour is extracted as easily and completely as that in unheated protein will be described later.

SUMMARY

(1) In order to investigate the fitness of water-dispersible nitrogen of heat-treated soybean flour as a criterion of the degree of denaturation, a method for determining the amount of native protein in aqueous solution which was based on the increase of susceptibility to protease action of *Asp. sojae* prior and after heat treatment was established.

---

[19] S. Mizushima and S. Akabori, "Chemistry of Proteins Vol. 2", Kyoritsu Press, Japan, p. 550.

(2) In the autoclaving of flour, there existed a minimum of water-dispersed nitrogen with regard to the heating time or the water content of flour. The higher the temperature of treatment or the water content of flour, the larger was the increase of dispersed nitrogen beyond the minimum.

(3) The higher the temperature or the water content of flour, the easier denaturation occurred. The native protein in the water extract of heated flour disappeared at the minimum point of water dispersibility.

(4) The water dispersibility method served as a criterion of denaturation for the flour heated within the range where a minimum point of water dispersibility was attained. Within this range amounts of water-dispersed nitrogen ran paralell to the amount of the native protein content. However, this water dispersibility method is not suitable for comparison of the degree of denaturation of flours treated under different conditions.

Acknowledgement. The author wishes to acknowledge his indebtness to Prof. K. Sakaguchi and Prof. R. Sasaki of University of Tokyo for their kind guidance and encouragement throughout this work, and also to Assist. Prof. H. Takahashi, University of Tokyo, and Dr. F. Yoshida of this Institute for their valuable suggestions. He also wishes to express sincere thanks to Dr. M. Mogi, President of this Institute, for his support and encouragement.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 1, p. 15～21, 1959]

Studies on Soybean Proteins

Part II. A New Method for Quantitative Determination of the Degree of Denaturation of Protein in Soybean Flour*

By Danji FUKUSHIMA

Noda Institute for Scientific Research, Noda-Shi, Chiba-Ken, Japan

Received April 1, 1958

Owing to the inadequacy of the water dispersibility method as a criterion for denaturation of the protein in soybean flours, a new method was devised which was quantitative and applicable to soybean flours denatured under various conditions. In this method the native protein in flour is determined by the quantity of protein which is solubilized but remains unhydrolyzed by the protease action of *Asp. sojae*. Conditions for the determination were decided as the result of various experiments.

It was found that the decrease in the amount of water-extractable protein in heated flour with low water contents was attributable to the prevention of extraction of native protein by partly denatured protein.

INTRODUCTION

Beckel et al.[1] had expressed the degree of denaturation by heat and moisture quantitatively, using water dispersibility of the nitrogen compounds of soybean oil meal as a criterion. However, this expression was inadequate and not universal, because it was shown in the previous report that water dispersibility is applicable as a criterion only upon meals denatured under extremely limited conditions and also that it was not quantitative.

A satisfactory method for determining the degree of denaturation of soybean meal has so far been reported. The author previously reported a method for determining native protein in water extract of soybean meal by protease action, but has not investigated whether or not the degree of denaturation of the flour is indicated by the amounts of the native protein contained in the water extract. In the present paper, this point was investigated precisely and a new method was proposed for the determination of denaturation of soybean. This method is quantitative for determining the amounts of native protein in a variety of heat-treated flours.

METHOD

Sample Preparation The defatted soybean flour which was used in this paper was the same as previously reported. It showed the following percentage composition: moisture, 7.01; nitrogen, 8.045. Heat treatment was also carried out by the same procedure. Heating of flours without the addition of water was carried out in the atmosphere at a definite temperature.

Protease Solution and Reagents

Protease Solution: The water extract of wheat bran koji of *Asp. sojae* KS was diluted by distilled water so that 10 ml of the diluted solution had an activity of $65[\mathrm{PU}]^{\mathrm{cas.\ 30°FR}}_{\mathrm{m.\ eq.\ tyr.}}$ at pH 7.2. The method of measurement of enzymatic activity has previously been described.

Reagents:

Buffer Solution ....One half and 0.25 mol. solution of phosphate buffers (pH

---

* Presented at the meeting of the Agricultural Chemical Society, Tokyo, September 28, 1957.

[1] A. C. Beckel, W. C. Bull and T. H. Hopper, *Ind. Eng. Chem.*, **34**, 973 (1942).

	7.2) was prepared.
Trichloroacetic Acid....	A 2.4 mol. solution was used.
Gum arabic	A two per cent. solution was prepared.
Toluol	This was used as preservative.

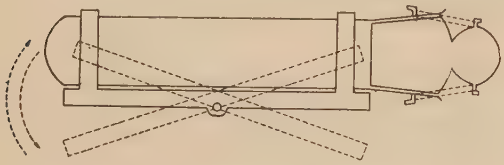


FIG. 1. Apparaturs of Digestion.

Apparatus For digestion by the protease, the apparatus shown in Fig. 1 was used. The tube had a capacity of about 25 ml. In this tube, the sample was put in, the enzyme solution and other reagents were added and it was stoppered tightly with grease and rubber strips. Then the mixture was placed in a water bath at 30°C and shaken at the rate of 60 r.p.m.

Procedure The flour sample, corresponding to 1g of flour before heat treatment, was transferred with 10 ml of 0.5 mol. phosphate buffer into the tube. Ten ml of the enzyme solution and 1 ml of toluol were added successively. After being stoppered, they were shaken for twenty hours at 30°C, as described above. At the end of this period the digestion mixtures were transferred to a graduated flask and made up to 50 ml with 0.25 mol. phosphate buffer. The resulted mixture was centrifuged for twenty minutes at 12,000 r.p.m. One ml of each supernatant was transferred into two test tubes (A and B). To tube A, 6 ml of distilled water, 2 ml of 2% gum arabic solution, and 1 ml of 2.4 mol. trichloroacetic acid were added successively and mixed. To tube B, 9 ml of 0.25 mol. phosphate buffer was added and mixed. Tube A was incubated for twenty minutes at 30°C, after which it was shaken vigorously. Three minutes later the absorption of the solution in tube A was read against that in tube B using a Hitachi EPO-B photometer with a 660 mμ filter. Shaking of the tube was necessary to obtain reproducible data, otherwise the substrate which was solid, has to be kept in a locally-concentrated state during digestion time.

EXPERIMENTAL

I. Extraction of Native Protein by Protease

If the native protein in the flour was completely extracted by water, regardless of the conditions of denaturation of the flour, the amounts of the native protein in water extract served as a criterion for the degree of denaturation of the flour. The method reported previously, by which the native protein in the aqueous solution could be measured would be applicable for the flour itself as well as for its water extract. The protease solution of *Asp. sojae* was added to the flours which had been autoclaved for various periods at 120°C without being added water. It was found that large amounts of protein were solubilized but not hydrolyzed in the solution. The amounts of the protein solubilized but not hydrolyzed after twenty hour's digestion at 30°C were measured by the turbidity method as shown in Fig. 2. As shown in Fig. 3, the amount of nitrogen compounds extracted from the flours heat-treated under these conditions by 1/4 mol. phosphate buffer was very little. This could not be attributed to the effect of salt solution, because water extraction gave almost the same extent of solubilisation. Therefore, it appeared that there existed a protein which could not be extracted without the addition of protease.

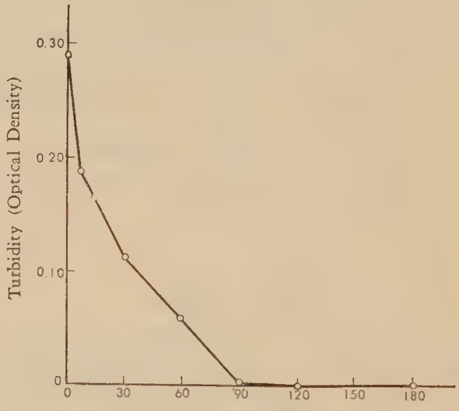


FIG. 2. Extraction of Protein in Soybean Flour Autoclaved at 120°C without Addition of Water by Protease Action.

The condition of digestion is the same as "Method".

The following experiment was performed to ascertain whether the solubilized but unhydrolyzed protein was native. For this purpose, the method reported previously in which the amounts of the native protein in the solution were determined was modified and applied to the solution of the digestion mixture, as described below.

The digestion mixtures were centrifuged and the supernatants were divided into two parts, one which was not heated and the other heated at 100°C for ten minutes. One ml of each portion was pipetted into a test tube. One ml of the solution of alkaline protease prepared from the culture medium of *Asp. sojae* by the method of Mizunuma et al.[2] was added to each tube. Initial activities of the protease solution added to each had been regulated beforehand so that both digestion mixtures had an activity of $10[\mathrm{PU}]^{\mathrm{cas.\ 30°FR}}_{\mathrm{m.\ eq.\ tyr.}}$. After thirty minute's digestion at 30°C the amount of the trichloroacetic acid-insoluble fraction was measured by the turbidity method. The difference between the two indicated the amounts of the native protein in the solution. The results are shown in Table I. On the other hand there existed only little or no native protein which was extracted by water (Table III). In spite of the fact that there was no native protein in the flour autoclaved for thirty minutes under these conditions, large amounts of native protein were extracted by protease action. Accordingly, the measurement of the denaturation degree of the flour by estimating the native protein in the water extract was meaningless. Therefore the native protein in the flour should first be solubilized by protease action. If adequate conditions for the protease action were chosen, the native protein could be measured. The following examination was performed to find out favorable conditions.

TABLE I. NATIVE PROTEIN EXTRACTED BY PROTEASE ACTION FROM SOYBEAN FLOUR AUTOCLAVED AT 120°C WITHOUT ADDITION OF WATER

Time of Autoclaving (min.)	Protein Extracted by Protease Action (optical density)	Difference (Amount of Native Protein) (optical density)
15	0.169	0.073*
30	0.120	0.065
60	0.055	0.024

* The true value is smaller than this, because of occurrence of precipitation by heat treatment

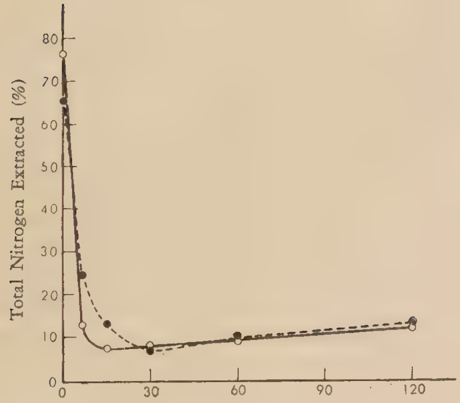


FIG. 3. Water- or Salt-Dispersibility of Soybean Flour Protein Autoclaved at 120°C without Addition of Water.

—○— Water
—●— 1/4 mol. phosphate buffer (pH 7.2)

In this article, "native protein" indicates the protein which showed an increased susceptibility to protease action after heat treatment in an aqueous state though hardly hydrolyzed before the treatment.

II. Digestion Time

In view of the susceptibility of the soybean flour protein to the protease action, two cases should be considered. The one is the increase of the susceptibility by heat treatment and the other the decrease. The former is the "denaturation" described previously in which denatured protein was usually insoluble in water. However, when the soybean flour was heated under particular conditions, the denatured protein was partly soluble in water. On the other hand, a decreased susceptibility was observed upon the flour autoclaved at a high temperature for a long period. This phenomenon was usually explained by the Melanoidin reaction that is the reaction between proteins and sugars to form complexes. The complexes were, however, considered to be hardly soluble in water. In the present method the "native protein" which was solubilized but remained unhydrolyzed by protease action, was measured. Therefore, the protein which was denatured and insolubilized after heat treatment did not have affect to this method. The denatured but solubilized protein which could be activated by the enzyme, had influence on determination of the native protein by this method. Besides these, native but insolubilized protein described in paragraph I, also had influence on this method. If digestion time was too short, there was a possibility that the native but insolubilized protein is not completely extracted, as well as the possibility of the denatured but solubilized protein being left in the solution without being hydrolyzed completely. On the other hand, if digestion was extended for a period too long, the accuracy of measurement lowered, because the native protein was hydrolyzed gradually, as had been shown in the previous report.

---

[2] T. Mizunuma and N. Iguchi, This Bulletin, **22**, 35 (1958).

From these considerations, the duration of digestion was examined and decided to be twenty hours. This period of digestion was quite reasonable as shown in the following three experiments. Firstly, the flours autoclaved under conditions where the denatured but solubilized protein was produced in large quantities, were digested. The denatured but solubilized protein was hydrolyzed completely during this period (Table IV). Secondary, the flours autoclaved under the condition (at 120°C without added water) where the native but insolubilized protein is produced in large quantities, were digested for twenty hours. The resulted mixtures were centrifuged off and the precipitates were digested once more. Any protein was not solubilized from the precipitates thus showing that the extraction of the native but insolubilized protein is completed during the above digestion period. Finally, as shown in Fig. 4, when heat-treated flour was used as the sample, approximately 70% of the total protein of the flour was hydrolyzed after twenty hour's digestion time. When native flour was used, only about 20% of the total protein was hydrolyzed under the same conditions. From the above three experiments, it was decided that a digestion of twenty hours was most suitable for this method.

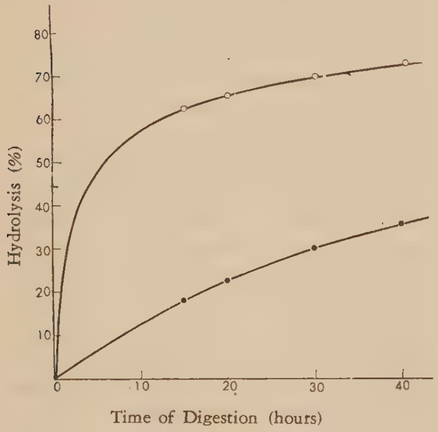


FIG. 4. Hydrolysis of Soybean Flour Protein by Protease of *Asp. sojae*.

—○— Treated at 100°C for 30 min. under the addition of 5-folds of water
—●— Not treated
Enzyme and Conditions of Digestion: The same as the "Method" in this paper.
Degree of Hydrolysis: Trichloroacetic acid-soluble nitrogen was measured.

III. Examination of Quantitative Relationship

(1) Relationship between Turbidity and Protein Concentration: In order to obtain quantitative results the turbidity appearing after the addition of trichloroacetic acid to the protein solution should be proportional to the protein concentration. The proportional relationship was obtained only when gum arabic was not put into a blank (Fig. 5). Although the gum arabic solution was somewhat opaque, the error resulting from the omittion of gum arabic to the blank was almost negligible in this measurement.

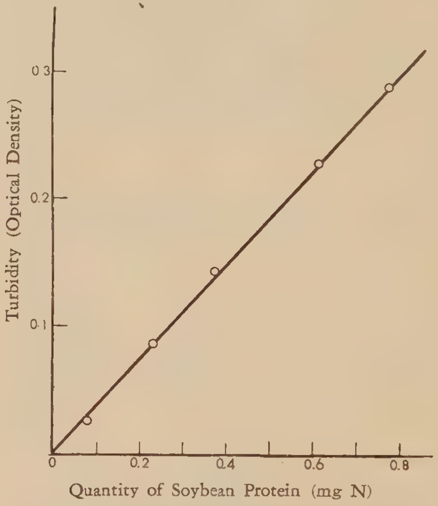


FIG. 5. Relationship between Turbidity and Soybean Protein Concentration.

(2) Relationship between Turbidity and Native Protein Quantity in Flour: It was subsequently examined whether or not the turbidity method applied to the flour was proportional to the native protein content of the flour. At the same time the effect of the digestion period was also examined. The native flour and the completely denatured flour were mixed with various ratios and 1g of each of these mixtures was digested for five, twenty, and forty hours, respectively. As shown in Fig. 6, the relationship between turbidity and protein content was essentially linear, regardless of the length of digestion time.

IV. Determination of Degree of Denaturation of Protein in Heat-Treated Soybean Flour

The amounts of the native protein in the heat-treated flours were determined by this method (Tables II, III, and IV). The water-dispersed nitrogen of these flours

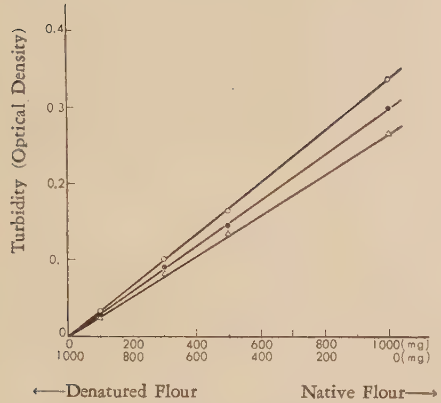


FIG. 6. Relationship between Turbidity and Native Protein Quantity in Soybean Flour.

—○— Five hours in digestion time
—●— Twenty hours
—△— Forty hours

TABLE II. WATER-DISPERSED NITROGEN, WATER-EXTRACTED NATIVE PROTEIN, AND TOTAL NATIVE PROTEIN IN FLOUR AUTOCLAVED UNDER ADDITION OF 100 PERCENT WATER

Temperature of Autoclaving	Time of Autoclaving (min.)	Water-dispersed Nitrogen (%)	Water-extracted Native Protein (γ Tyrosine)	Total Native Protein in Flour (Turbidity)
	Native	74.4	9.6	0.301
100°C	7	28.2	2.9	0.146
	15	27.0	2.7	0.137
	30	24.1	2.5	0.114
	60	18.6	1.0	0.080
	120	18.6	0	0.023
	180	19.6	0	0.002
110°C	7	13.6	0	0
	15	12.7	0	0
	30	13.7	0	0
	60	17.0	0	0
	120	20.1	0	0
	180	24.5	0	0
120°C	7	13.9	0	0
	15	13.7	0	0
	30	14.7	0	0
	60	19.4	0	0
	120	24.4	0	0
	180	29.8	0	0

TABLE III. WATER-DISPERSED NITROGEN, WATER-EXTRACTED NATIVE PROTEIN, AND TOTAL NATIVE PROTEIN IN FLOUR HEATED AT 120 DEGREE CENTIGRADE

Water Content of Flour before Heating and Heating Procedure	Time of Heating (min.)	Water-dispersed Nitrogen (%)	Water-extracted Native Protein (γ Tyrosine)	Total Native Protein in Flour (Turbidity)
	Native	77.0	9.7	0.302
P_2O_5-Dehydrated and Dry Heating	60	72.6	—	0.281
	120	66.4	—	0.275
	180	60.8	—	0.252
	240	46.7	—	0.240
	300	34.9	—	0.229
Non Water-Added and Autoclaving	7	14.9	1.4	0.186
	15	7.7	0.5	0.166
	30	7.4	0	0.104
	60	9.1	0	0.060
	90	12.0	0	0.005
	120	14.5	0	0
	180	20.1	0	0
100% Water-added and Autoclaving	7	13.9	0	0
	15	13.7	0	0
	30	14.7	0	0
	60	19.4	0	0
	120	24.4	0	0
	180	29.8	0	0

and the native protein in their water extracts is also shown in the Tables.

By autoclaving at 100°C under a 100%-addition of water, a small amount of native protein still remained in the flour after three hour's heating. However, the native protein completely disappeared after a few minutes heating at 110°C and 120°C (Table II). On the other hand the native protein in the water extract disappeared after heating for two hours at 100°C (Table II). It was observed that an insolubilization of the native protein not being due to denaturation slightly occurred by heat treatment at 100°C under 100%-addition of water.

When the flour was autoclaved without any addition of water, the denaturation did not easily occur even at 120°C. And, the native protein in the flour did not disappear, until the flour was heated for ninety minutes (Table III). On the other hand, the native protein in the water extract disappeared, by heating for thirty minutes. At this heat-time, 35% of the

Table IV. Water-dispersed Nitrogen, Water-extracted Native Protein, and Total Native Protein in Flour Autoclaved at 100 Degree Centigrade for Thirty Minutes

Amount of Water Added to Flour as Autoclaved (cc)	Water-Dispersed Nitrogen (%)	Water-Extracted Native Protein (γ Tyrosine)	Total Native Protein in Flour (Turbidity)
Native	74.4	9.6	0.301
0	27.9	4.9	0.186
0.1	23.9	4.1	0.173
0.3	23.6	3.8	0.166
0.5	25.3	3.0	0.160
1.0	20.0	2.8	0.100
2.0	15.6	0	0.004
4.0	34.5	0	0
7.0	57.3	0	0
10.0	64.3	0	0

initial native protein in fact, was remaining in the flour. This indicated that the insolubilization of the native protein occurred in a greater extent under these conditions. The water dispersibility of the nitrogen of this flour was lower than that of the flour autoclaved at the same temperature with a 100%-addition of water. Therefore, the water dispersibility did not serve as a criterion for the degree of denaturation.

The flour which had been dehydrated for several days at room temperature in a vaccum desicator containing P_2O_5, was heat-treated at 120°C without the addition of water. It was found that both the native protein and the water-dispersed nitrogen decreased together with heat time. But the decrease of water-dispersed nitrogen was more remarkable than that of the native protein (Table III). Therefore the native but insolubilized protein tended to increase under a long dry heating.

By autoclaving the flour with definite amounts of water at 100°C for thirty minutes, the native protein in both the flour and its water extract disappeared completely at a point where the extracted nitrogen reached its minimum level: this occurred in the flour which contained a 200%-addition of water (Table IV). Presumably, the insolubilization of the native protein which was not due to denaturation did not occur in the presence of 200% of water.

DISCUSSION

This method determined a protein which was soluble in water and hardly hydrolyzed by the protease action of *Asp. sojae*. Soybean flour consisted of various components of the protein. Some of them were insoluble in water and some were easily hydrolyzed by protease action in the native state. Therefore, the absolute degree of denaturation could not be measured by this method, because soybean flour contained various protein. However, more than 60%[*] of the total proteins in soybean flour were water-soluble proteins, whose susceptibilities to protease action remarkably increased upon heat treatment. Accordingly, the degree of denaturation of soybean proteins could be represented by those proteins.

Of particular significance was the discovery that the native protein was not extracted by either water or buffer solution (Fig. 3), when the protein in the flour was partially denatured under low water contents. It was not examined whether or not this native but insolubilized protein was partly modified, though the susceptibility to the protease action after treatment could not be distinguished from unheated protein. A most probable explanation for this phenomenon might be that the heat-treated flour particles with a low water content, covered by the denatured and insolubilized protein, and the remaining native protein constituted a core of its particle. Thus, the particle seemed to behave insoluble before protease action, by which the insoluble membrane of the particles was solubilized by the enzyme, and the core-native protein came out into the solution. The observation on the appearance of trichloroacetic acid-soluble nitrogen compound prior to that of the native protein during the digstion of particles was indicative. Of course, other possibility could not be excluded. According to such a phenomenon, the water dispersibility method should not be applied to flour, which were heat-treated with low water contents. In the meanwhile, it had been discussed in the previous report that the method based on protease action directly indicated a change of the configulation of protein molecules, differing from the procedures by the dispersibility of protein in water, salts, or alkali solution. Therefore the method described here should be regarded to be most suitable for measuring the protein in soybean flour itself.

---

[*] This value was calculated from the amounts of protein measured by turbidity in this method.

SUMMARY

(1) The majority of soybean proteins were hardly hydrolyzed in native state by the protease of *Asp. sojae*, but became susceptible to the enzyme upon heating in a solubilized state. From this fact the method for determining the native protein in the soybean flour was investigated.

(2) The insolubilization of the protein, which occurred by heating under low water contents, was not attributed to its denaturation. The native protein in these flour was not extracted by water or buffer solution, but could be solubilized after the protease action. Therefore the measurement of the native protein in the water extract by the method previously described was inadequate for the purpose of measuring a degree of denaturation of heat treated flours.

(3) A new method was described, which was quantitative and applicable to the flours heated under various conditions. In this method the native protein in the flours was determined by applying the turbidimetric method to proteins which were extracted but unhydrolyzed after a twenty-hour digestion (30°C, pH 7.2) of flours by the protease of *Asp. sojae* with an activity of 65 $[\mathrm{PU}]^{\mathrm{cas.\ 30^{\circ}FR}}_{\mathrm{m.\ eq.\ tyr.}}$ at pH 7.2.

Acknowledgement. The author wishes to acknowledge his indebtness to Prof. K. Sakaguchi and Prof. R. Sasaki of University of Tokyo for their kind guidance and encouragement throughout this work, and also to Assist. Prof. H. Takahashi of University of Tokyo and Dr. F. Yoshida of this Institute for their valuable suggestions. He also wishes to express sincere thanks to Dr. M. Mogi, President of this Institute, for his support and encouragement.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 1, p. 22~28, 1959]

Studies on the Activities of Bacteria in Soy Sauce Brewing

Part IV. Proteases, Transaminases of *Pediococcus soyae*, nov. sp., and its Numerical Transition in Soy Mash

By Kenji SAKAGUCHI

Noda Institute for Scientific Research

Received June 30, 1958

The extracellular proteinase and peptidase of *Pediococcus soyae*[1] is not found in its growing culture solution and culture filtrate. In the cell extract, weak activities of proteinase and peptidase are observed.

Weak transaminase and reductive amination activities of producing glutamic acid from α-keto-glutaric acid are estimated in the resting cell suspension and also in the crude cell extract. The potent amino group doners are; alanine, leucine, lysine, aspartic acid and ammonium chloride.

Starch fermentation by *Ped. soyae* strain *d*6 is confirmed.

The numbers of lactic acid bacteria in soy koji are about 0.1–3 billion per gram and are much numerous than aerobic spore forming bacteria. The majority of them are salt sensitive *Streptococci* and *Lactobacilli*, but fewer salt tolerant *Pediococcus soyae* also exist. In the soy mash, salt sensitive lactic acid bacteria decrease rapidly within a period of two months. On the contrary, *Ped. soyae* grows well in the 18% salted mash, starting at 10^2–10^3/ml, reaching to 10^8–10^9/ml at the 4th month of brewing. Thereafter the viable counts of *Ped. soyae* gradually decreased and settled down to about 10^4/ml after 9 months of brewing. The pH value in soy mash was lowered while *Ped. soyae* was 10^6/ml or more.

Soy sauce brewing is very characteristic in microbiological industry because the principal biochemical reaction is the proteolysis of vegetable proteins, subsequently many reactions concerning peptides and amino acids. In the previous paper,[1,2] it was concluded that the most active bacteria in soy mash is alactic acid bacterium i.e., *Pediococcus soyae* nov. sp.. It is generally admitted that the proteolytic enzymes of lactic acid bacteria is not recognized or very weak. Nevertheless, endocellular proteolytic enzymes have recently bean detected in several lactic acid bacteria. Berger[3] reported on the properties of intracellular peptidases of *Leuconostoc mesenteroides*. Nelson and his colleagues have further investigated the proteolytic enzymes of cheddar cheese originating in the extracellular and endocellular proteinases and peptidases of *Lactobacillus casei*[4] and *Streptococcus lactis*.[5] Other investigators have also conducted studies on these cheese proteinases.[6,7,8]

Concerning the transaminase activity of lactic acid bacteria, the existence of this activity has been reported on *Streptococcus faecalis*.[9,10,11] *Leuconostoc mesenteroides*[10] and *Lactobacillus ar-*

---

1) K. Sakaguchi, This Bulletin, **22**, 353 (1958).
2) K. Sakaguchi, This Bulletin, **22**, 345 (1958).
3) J. Berger, et al., *J. Biol. Chem.*, **124**, 395 (1938), *J. Bact.*, **36**, 521 (1938).
4) E. Brandsaeter and F. E. Nelson, *J. Bact.*, **72**, 68, 73 (1956).
5) W. C. van der Zant and F. E. Nelson, *J. Dairy Sci.*, **36**, 1212 (1953), **37**, 795 (1954).
6) A. T. Dudani, Ph. D. Thesis, Iowa State College Lib.
7) J. Christophersen and H. Thiele, *Kieler Milchw. Forschung ber.*, **4**, 683 (1953).
8) L. E. Babrio and M. M. Foster, *J. Dairy Sci.*, **35**, 149 (1952).
9) J. T. Holden et al., *J. Biol. Chem.*, **191**, 559 (1951).
10) A. Meister, *Adv. in Enzymol.*, **16**, 185 (1955).
11) A. Meister, *J. Biol. Chem.*, **195**, 813 (1952).

avinosus.[10,11] However, the experimental method applied in these reports was the replacing of certain amino acids with corresponding keto-acids in a complete synthetic medium in which test organisms were inoculated and growth responses examined. In the case of *Ped. soyae*, a peptide-like nutritional factor (P-factor)[1,21] is required for growth, and the replacing method cannot be applied to this. In this paper, attempts were made for the detection of extracellular proteinases and also to detect the cooperation with *Aspergillus* proteinases. The endocellular proteinase and peptidase, the transaminase of this organism were also studied. Starch fermentation was confirmed by a weak starch fermenting strain *Ped. soyae d* 6.[1]

This paper also diels with the species and the viable cell numbers of lactic acid bacteria in soy koji, and the numerical changes of salt sensitive and salt tolerant lactic acid bacteia throughout a full-year of soy mash brewing.

EXPERIMENTAL

Measurement of proteinase activity. An application of Anson's[12] method reported in the preceding paper[2] was adopted. The substrate for the proteolysis was Hammersten casein (Merck), which was enzymated at pH 6.5.

Extraction of Intracellular enzymes. Based on the reports of Brandsaeter and Nelson concerning endocellular proteases of *Lactobacillus casei*[4] an eight-day culture of *Ped. soyae dl* 2 or *d* 4 was centrifuged, the cells were harvested and washed 3 times with a phosphate buffer of pH 7.0. Then the cells were destroyed by a 10 KC sonic oscillator (Kubota) for thirty minutes and the cell debris were centrifuged by rotation of 6000 per minute, for thirty minutes. The supernatant could be stored for several days at −30°C in vacuum flask cooled with dry ice.

Measurement of peptidase activity. Two millilitres of the supernatant of *Ped. soyae* cell extract was added to 5 ml of the substrate solutions buffered with M/15 phosphate pH 6.5. The substrates were 400 μM of glycyl-L-leucine (Nutritional Biochemicals Corp.), L-alanyl-glycyl-glycine (Delta Chemical Works). The mixed solutions were incubated for ten hours at 37°C, and the liberated amino acids were titrated with a N/50 alcoholic solution of potassium hydroxide, following micro-alcoholic titration[4,13] by the method of Grassman and Heyde.

Measurement of transaminase activity.[14] In a total volume of 1.5 ml, 25 μM of α-keto-glutaric acid, 50 μM of each L-amino acid, 0.75 ml of pH 7.0 phosphate buffer, and 4.9 mg (as dry matter) of washed cell suspension or the corresponding volume of the maceration sap of cells were thoroughly mixed, and enzymated for four hours at 37°C. After lapse of enzymation period the flasks were placed into a boiling water bath for five minutes so as to arrest the reaction. The produced L-glutamic acid was measured manometrically using dried cells of *Escherichia coli* Najjar's strain[15].

Starch fermentation. *Ped. soyae d* 6[1] was subcultured for five days in the glucose supplemented nutrient broth, and then inoculated in the following broth: Soluble starch (Merck) 12 g, polypepton (Takeda) 3 g, beef extract (Difco) 1.2 g, dipotassium phosphate 2 g, salt 2 g, calcium carbonate 8 g and brom thymol blue. With occasional neutralization by concentrated sodium hydroxide solution, the inoculated broth was incubated anaerobically for four months at 30°C, and analyzed according to the routine method[1].

Viable counting of lactic acid bacteria in soy koji and in soy mash. One gram of the soy koji or 1 ml of the soy mash was suspended in 50 ml of sterilized glucose supplemented nutrient broth (For dilution of soy mash, salt was added to 17% in the broth.); 0.5 ml of the broth was pipetted and transferred aseptically to the next flask containing 50 ml of the broth. From the suitably diluted suspension, 0.5 ml was pipetted, inoculated into the isolation medium described in the previous paper, and cultured in test tube. In order to inhibit the surface growth of molds and aerobic bacteria, the uninoculated medium was poured into the tube after the inoculated agar became solid. The added antifungal antibiotic eurocidin inhibited the growth of molds and yeasts[20]. In order to count the viable numbers of *Ped. soyae*, the medium was salted to 15%. Viable numbers of salt-sensitive lactic acid bacteria were represented by the difference in colony counts between the 15% salted and the unsalted medium.

---

21) K. Sakaguchi, This Bulletin, in press (Part VII).

12) M. L. Anson, *J. Gen. Physiol.*, **22**, 79 (1938).

13) M. Grassmann and W. Heyde, *Z. physiol. Chem.*, **183**, 32 (1929).

14) I. Shiio et al., *J. Biochem.*, **43**, 779 (1956).

15) V. A. Najjar, *J. Biol. Chem.*, **175**, 281 (1948)

RESULTS

Proteolysis by *Pediococcus soyae*.

Detection of proteolytic activity in the growing culture. Ten strains of *Ped. soyae* isolated from the soy mash, and, for comparsion, *Lactobacillus arabinosus* and *Streptococcus faecalis* R were cultured in the following medium for twelve days at 30°C and the degradation of added milk casein was investigated. The medium composition was: Hammersten casein 1%, yeast extract (Difco) 0.5%, polypepton (Tadeda) 0.1%, glucose 2% and calcium carbonate 0.5%. The free amino group in the culture solution was estimated by Van Slyke's volumetric gasmetry. The nitrogenous compounds which were soluble in 0.4 M trichloroacetic acid were also determined by Kjeldahl's method.

Although the cheddar cheese lactic acid bacteria, *Lactobacillus casei*,[4] was reported to hydrolyse milk casein in growing culture, *Ped. soyae* did not suggest any proteolytic activity in the medium, and the added pepton was found to be only consumed. (Table I)

TABLE I. DECOMPOSITION OF MILK CASEIN BY GROWING CULTURE OF *Ped. soyae*

Strain	Free Amino N mg/ml (Van Slyke)	TCA soluble N mg/ml (Kjeldahl)
Control	0.47	1.40
d 1	0.38	1.30
d 2	0.30	1.20
d 3	0.37	1.25
d 4	0.35	1.30
d 5	0.44	1.25
d 6	0.34	1.35
d 7	0.37	1.35
d 9	0.25	1.35
dl 1	0.37	1.30
dl 2	0.39	1.35
L. arabinosus	0.35	1.20
Str. faecalis	0.36	1.35

Detection of proteolytic activity in the culture filtrate. The supernatnat (7000 r.p.m., for fifteen minutes) of the culture solution was used as the enzyme solution and proteolytic activity on the following substrates was examined: Hammersten casein, its partial hydrolysates by *Asp. sojae* proteinase, *Bacillus subtilis* crystalline proteinase (Nagase Ind.) and hydrochloric acid. Enzymation was continued for eighteen hours at 30°C with the addition of thymol and penicillin. There was no increase of phenolic amino acids on any substrates beyond the range of measurement error (Table II). Therefore, extracellular proteolytic enzymes of *Ped. soyae*, both proteinase and peptidase, were not detected at pH 6.0 and 4.0.

TABLE II. DETECTION OF EXTRACELLULAR PROTEINASE

Substrate	Increase of Optical Density	
	pH 4.0	pH 6.0
Casein	−0.005	−0.003
Partial Hydrolysate of Casein by *Asp. sojae* Protease	0.001	0.005
Partial Hydrolysate of Casein by Crystalline *Bac. subtilis* Protease	0.004	0.002
Partial Hydrolysate of Casein by 2 N HCl	0.005	0.003

The assumption that lactic acid bacteria, though impotent in proteolytic activities by themselves, may strengthen or support the proteolytic activity of *Asp. sojae* was also investigated. The culture filtrate of *Ped. soyae* was added to an equivalent volume of *Asp. sojae* mold bran extract (1 g per 20 ml) which was enzymated with Hammersten casein at pH 4.0 for four hours at 30°C. The results shown in Table III indicate that there is no significant difference discernible whether the culture filtrate of *Ped. soyae* is added or not.

TABLE III. DETECTION OF THE PROTEOLYTIC COOPERATIN WITH *Asp. sojae* PROTEINASE

Enzyme Solution	Increase of Optical Density	
	Example 1	Example 2
Ped. soyae	−0.009	−0.007
Crude Extract of *Asp. sojae* Mold Bran	0.047	0.165
Crude Extract of *Asp. sojae* Mold Bran + *Ped. soyae*	0.046	0.160

Endocellular proteinase and peptidases. Brandsaeter and Nelson reported on the protelytic strain of *Lact. casei*,4) and also on the existence of a rather weaker endocellular proteinase (opt. pH 5.0–7.0) and a stronger endocellular peptidases (opt. pH 6.0–8.0). Similar results of *Streptococcus lactis*5) were obtained by Van der Zant and Nelson. *Ped. soyae* is also a distinguished lactic acid bacterium in regard of playing a role in proteolytic fermentation, hence endocellular proteolytic enzymes are to be studied.

The cell free extract was filtered with Seitz's filter and 1 ml of the sterile filtrate was added to 2 ml of autoclaved Hammersten casein solution at 30°C, pH 6.5. The results shown in Table IV clearly indicate the existence of endocellular proteinase activity, but by reflecting on the concentration of the enzyme solution and the length of enzymation time the weakness of activity is affirmable.

TABLE IV. INTRACELLULAR RROTEINASE OF *Ped. soyae*

Incubation Days		Transmittance %	Optical Density	Increase of O.D.
1	Enzyme Solution	88.2	0.054	0.039
	Casein+Enzyme Solution	80.2	0.094	
7	Enzyme Solution	81.3	0.088	0.125
	Casein+Enzyme Solution	61.1	0.213	

The endocellular peptidases were estimated in the same manner as in the enzymation for ten hours at 37°C, pH 6.5. As shown in Fig. 1, about 20–25% of the substrates were splitted after ten hours enzymation. Activity was stronger than endocellular proteinase activity, but resulted to be weaker than the endocellular peptidases of *Lact. casei* in cheddar cheese.4)

Transaminase and glutamic acid dehydrogenase. Transaminase activity has been observed in many microorganisms.10,16) Among lactic acid bacteria, its existence in *Streptococcus faecalis*9,10,11), *Leuconostoc mesenteroides*,10) *Lactobacillus arabinosus*10,11) have been reported. In spite of this, the experimental method of most of these reports is as stated in the preface the replacing method which can't be applied to *Ped. soyae*. In this experiment, intact cell suspension and crude cell extract by sonic oscillation were employed as the enzyme solution.

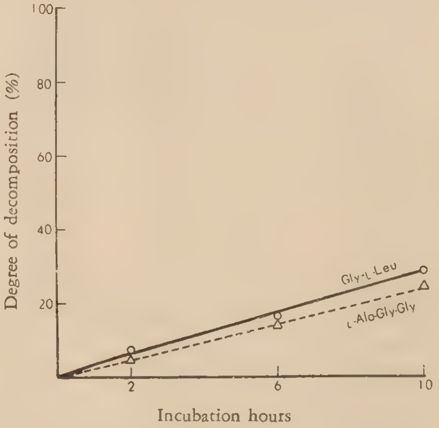


FIG. 1. Intracellular Peptidase of *Ped. soyae*

The relative values of the produced L-glutamic acid against added α-keto-glutaric acid are listed in Table V. Though the activities were in general low, rather broad specificities were observed in many amino acids. The potent amino group doners were: alanine, leucine, valine, histidine, lysine, tyrosine, aspartic acid, cystine and ammonium chloride. It is noteworthy that ammonium chloride was the most potent amino group doner, thus, the activity of glutamic acid dehydrogenase was presumed.

Soy mash is an almost saturated solution composed of amino acids, and the existence of transaminase activity in the most active bacteria, *Ped. soyae* provides an important suggestion for the glutamic acid production in soy mash. That is, another synthetic way for producing glutamic acid from sugar-materials is to be considered besides the hydrolytic way of protein-material. Nevertheless, the real action of *Ped. soyae* transaminase in soy mash

---

16) H. Katagiri, et at., read at the General Meeting of Agr. Chem. Soc. Japan, on 4th, May, 1958.

TABLE V. TRANSAMINASE ACTIVITY OF *Ped. soyae*

Doner of amino group	Relative value of L-glutamic acid formed: Intact cell %	Relative value of L-glutamic acid formed: Crude extract %
glycine	0.1	1.3
alanine	0.3	4.0
isoleucine	1.1	2.0
leueine	1.0	2.9
valine	1.2	2.3
arginine	1.2	1.7
histidine	1.7	2.0
lysine	1.3	3.0
serine	1.0	2.3
threonine	1.2	2.3
phenyl alanine	1.1	0.0
tyrosine	1.5	2.4
tryptophane	0.6	−0.5
aspartic acid	1.0	3.9
proline	0.1	1.9
cystine	0.0	2.6
cysteine	−0.3	0.2
methionine	−0.1	−0.2
NH_4Cl	1.4	4.3

is doubtful because of its low pH value and high salt content. In the author's unpublished data,[17] about 30% of α-keto-glutaric acid added to the one-year old brewed soy-mash was converted into L-glutamic acid with the addition of ammonium phosphate. This phenomenon was however, not observed in the fresh soy mash in which *Ped. soyae* was actively growing. Therefore, it is probable that either yeasts or any other unidentified microorganisms might play a role in the transamination of soy mash.

Starch fermentation by *Ped. soyae* strain *d* 6. In the previous paper, *Ped. soyae* strain *d* 6 and *dl* 1 revealed weak starch fermentability after one moth's anaerobic incubation at 30°C. The starch fermentation by lactic acid bacteria was rarely observed except that by the well-known starch fermenters *Streptococcus bovis*[18] and *Str. equinus*.[19]

Fermentation began after one month's lag period and thereafter smoothly proceeded until almost all of the added starch was consumed, which was after four month's fermentation. From 10.9 g of consumed starch, 1.44 g of ethanol, 3.55 g of acetic acid and 4.28 g of dextrorotatory lactic acid were produced. Fermentation tests were also conducted on 7% and 15% salted broth, but no growth was observed in both cases. Amylase tests for this strain were negative on both α-amylase and β-amylase activity.

Lactic acid bacteria in soy koji. In order to investigate the source and the infecting route of *Ped. soyae* into soy mash, lactic acid bacteria in soy koji were isolated and viable numbers per gram were counted.

The addition of an antifungal antibiotic, eurocidin,[20] could effectively inhibit the growth of the koji mold (*Asp. sojae*) and contaminating yeasts. The tube culturing in which a low rH value was retained by the addition of sodium thioglycollate, only permitted the growth of lactic acid bacteria amongst other micoorganisms. The tube culture with the aid of 0.1% sodium thioglycollate provided a more perfect anaerobic condition than plate culturing in a vacuum jar, and resulted in much more colony counts of lactic acid bacteria.

Viable numbers of lactic acid bacteria in soy koji manufactured in the factory of Noda Shoyu Co. were from 0.1 to 3 billion per gram, and about ten-fold more than the viable counts of the *Bacillus subtilis* group, which were formerly accepted as the main contaminants in soy koji or rice koji. From the colonies grown in the tubed agar, 46 strains were isolated and inspected microscopically.

Gas producing lactic acid bacteria were few, and short-chained short rods (0.3 μ × 1.0 μ, supposed to be a *Lactobacillus* sp.,) and short-chained cocci (0.5–0.7 μ in diameter, supposed to be a *Streptococcus* sp..) prevailed. These two types of lactic acid bacteria could grow only in 7% salted medium, but not in 15% salted

---

17) K. Sakaguchi, unpublished.

18) Shermen, *Bact. Rev.*, **1**, 57 (1937).

19) Andrewes and Horder ,*Lancet*, **2**, 712 (1906)

20) K. Sakagushi, *J. Agr. Chem. Soc. Japan*, **28**, 758 (1954); *Rep. Noda Inst. Sci. Res.*, **2**, 25, (1958).

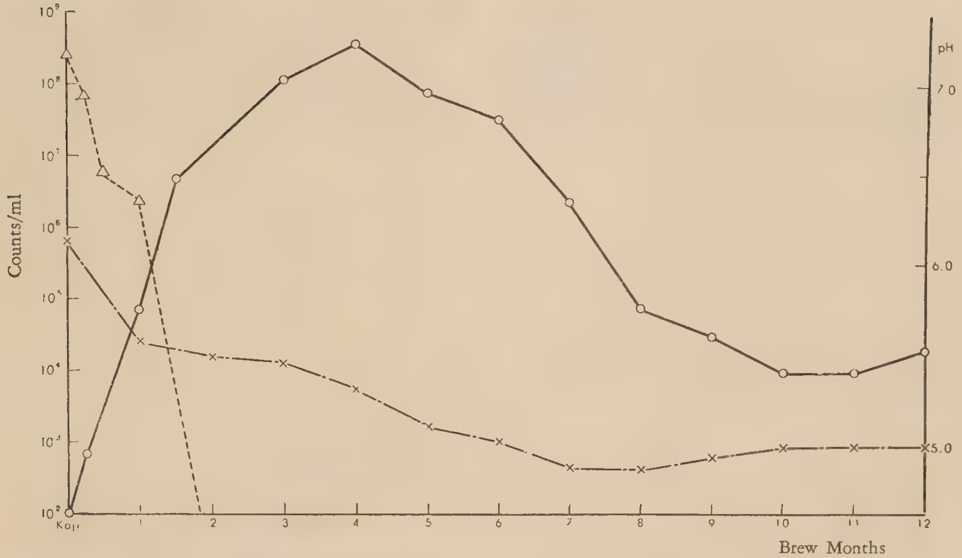


FIG. 2. Change of Viable Counts of Lactic Acid Bacteria in Soy Mash
——O—— *Pediococcus soyae* ----△---- Salt sensitive lactic acid bact. ----×---- pH

medium. The two salt-senstive strains isolated from fresh soy mashes[1] had identical forms with those in soy koji.

Four strains of tetra formed *Pediococci* were found from the isolated strains, and grew well in 15% salted medium. It is therefore concluded from these experiments that the origin of *Ped. soyae* in soy mash is primarilly due to soy koji, which is probably originated to the covering microbial atmosphere for a period of three hundred years' soy sauce brewing in Noda City.

Numerical transition of *Ped. soyae* and other lactic acid bacteria in soy mash. On the soy mash collected from the Seventh Factory of Noda Shoyu Co. throughout one years brewing, changes of viable counts in *Ped. soyae* and salt sensitive lactic acid bacteria were examined. The number of *Ped. soyae* was represented by the colony counts grown in 15% salted agar. The number of salt sensitive lactic acid bacteria was represented by the difference of colony counts between salted the unsalted media.

Fig. 2 gives the result. Salt-sensitive lactic acid bacteria grown in the soy koji diminished in their viable numbers, but about 10^6–10^7 cells per millilitre survived within one month's brewing in a 18% salted circumstance. These did not multiply, but their physiological activities may still have been active depressing the pH of the soy mash. After one and a half month's brewing at about 30°C, *Ped. soyae* exceeded in numbers, while salt-sensitive bacteria diminished and the pH of the mash was lowered due to the rapid growth of the organism. On the fourth month of brewing, viable numbers of *Ped. soyae* reached to its maximum, namely 260 million per millitlitre. Thereafter colony counts slowly decreased, and settled down to about 10^4 counts per millilitre. The pH of the mash was minimum on the seventh or eighth month reaching to pH 4.85 (measured with a glass electrode pH meter). When the count of viable numbers of *Ped. soyae* was 10^5/ml or less, the pH of the soy mash gave rise and came up to pH. 5.0 at the end of brewing.

Acknowledgements. The author wishes to express his hearty thanks to Prof. T. Asai and

Prof. K. Kitahara for their kind guidances. Thanks are also due to Dr. K. Sakaguchi, Prof. Y. Sumiki, Mr. A. Ōbayashi, Dr. M. Mogi and members of this institute for their valuable suggestions. The author is extremely grateful to Mr. T. Kuramochi for his able assistance in this study.

A part of this report was read at the General Meeting of the Soc. Agr. Chem. Japan on 3rd, May, 1958.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 1, p. 28～33, 1959]

Studies on Phyto-peroxidase

Part XII. Amino Acid Composition of Japanese-radish Peroxidase a*

By Yuhei MORITA and Katsuzo KAMEDA

Research Institute for Food Science, Kyoto University

Received July 1, 1958

The amino acid composition of hydrolysates of crystalline Japanese-radish peroxidase a has been determined mainly by chromatography on columns of Dowex 50–X8. The recoveries of the residues and the nitrogen were 93.52 and 100.90 per cent respectively, both including those of the carbohydrate residues and protohematin. The analyses indicate the presence of the following 384 amino acid residues, 65 carbohydrate residues and 1 protohematin in the molecule of peroxidase a (mol. wt. 54500): Asp$_{51}$Glu$_{26}$Gly$_{6}$Ala$_{64}$Val$_{20}$Leu$_{36}$Ileu$_{15}$Ser$_{51}$Thr$_{27}$(Cys-)$_{10}$Met$_{4}$Pro$_{17}$-Hypro$_{12}$Phe$_{18}$Tyr$_{3}$Try$_{2}$His$_{4}$Lys$_{7}$Arg$_{11}$(-CONH$_{2}$)$_{50}$Mannose$_{35}$Xylose$_{11}$Arabinose$_{4}$Hexosamine$_{15}$Protohematin$_{1}$.

INTRODUCTION

The authors have prepared crystalline peroxidase a from the roots of Japanese-radish (*Raphanus sativus L.*), and studied on some of its enzymic, chemical and physical properties$^{1\sim6)}$. In the previous investigation, the authors have found that this enzyme was a glycoprotein, containing mannose, xylose, arabinose, and hexosamine, and from titrimetric experiments suggested that some groups in the enzyme were in a masked state against protons. In connection with these results, it is important to acquire a thorough knowledge of the amino acid composition of this enzyme. However, so far there is no study on the complete amino acid composition of peroxidases, though partial and qualitative information on the crystalline horseradish peroxidase II was obtained by Theorell and his coworkers$^{7,8)}$.

---

* Presented at the meeting of the Agricultural Chemical Society of Japan, Kyoto, May 3, 1958.

1) Y. Morita and K. Kameda, *Mem. Research Inst. Food Sci., Kyoto Univ.*, **12**, 14 (1957).

2) Y. Morita and K. Kameda, *ibid.*, **14**, 49 (1958).

3) Y. Morita and K. Kameda, *ibid.*, **14**, 61 (1958).

4) Y. Morita and K. Kondo, *Bull. Research Inst. Food Sci., Kyoto Univ.*, **13**, 60 (1954).

5) Y. Morita, *ibid.*, **15**, 64 (1954).

6) Y. Morita and K. Hasegawa, *ibid.*, **15** ,82 (1954).

7) H. Theorell and Å. Åkeson, *Arkiv Kemi, Mineral. Geol.*, **17B**, No. 7 (1943).

8) A. C. Maehly and S. Paléus, *Acta Chem. Scand.*, **4**, 508 (1950).

This lack of knowledge on the amino acid composition of peroxidase would be due to the presence of carbohydrate in the enzyme, which produces considerable amounts of humin by the ordinary acid-hydrolysis under aerobic condition.

This paper presents a study of the amino acid composition of the Japanese-radish peroxidase a which was evaluated mainly by chromatographic separation of the amino acids on the sulfonated polystyrene resin, Dowex 50, and by their quantitative estimation with the ninhydrin reaction following the procedure of Moore and Stein[9,10]. A fairly satisfactory estimation was achieved under an anaerobic condition of hydrolysis for various durations.

MATERIAL AND METHODS

1. Japanese-radish peroxidase a. The Japanese-radish peroxidase a was obtained in the crystalline form and recrystallized with ammonium sulphate by the procedures described in the previous report[1]. This preparation was found to be homogeneous by electrophoretic analysis and it contained 12.73% nitrogen and 1.166% protohematin. The Reinheit Zahl and the purpurogalline number were 3.28 and 1420 respectively. Prior to the analyses, the sample was dialyzed against deionized water for three weeks at 0°C to remove the salts, and then de-hematinized by cold acid acetone according to the method of Gjessing and Sumner[11] as described in the previous report[2]. This-apo-peroxidase was dried in vacuo over pellets of sodium hydroxide for twenty four hours, and separate aliquots were weighed out for hydrolysis and for the determination of moisture. Moisture was determined by drying to constant weight at 105°C, and the nitrogen content was determined by the semi-micro Kjeldahl method.

2. Hydrolysis. Each hydrolysis was performed on about 7 mg of apo-peroxidase in a sealed glass tube. After the protein was dissolved in 2 ml of 6N HCl, the solution was frozen in a bath of solid carbon dioxide and ethanol. The tube was once evacuated with an oil pump to replace the air with argon as the inert gas, and sealed. In all experiments reported herein, hydrolysis was performed in sealed tubes under an argon gas phase. In preliminary work, the hydrolysis was performed in the presence of air. This resulted in the formation of large amounts of insoluble black humin (about 3% in dry basis), while, under the argon gas phase, the formation of humin was markedly less than under the aerobic condition. Hydrolyses were performed in an oven at 104°C for twenty two or seventy hours. After hydrolyses, the cooled tubes were opened and their contents, being light yellow and containing a trace of humin, were evaporated to dryness over pellets of sodium hydroxide in vacuo at 4°C. Each residue was dissolved in 0.5 ml of the initiated buffer solution made slightly acidic with 0.1 ml of 0.1N HCl, and chromatographed.

Independent determinations of cystine and cysteine as cysteic acid were performed by the method of Schram, Moore and Bigwood[12]. Four separate aliquots of apo-peroxidase in glass tubes were oxidized with performic acid prior to hydrolysis. To 7 mg of protein, were added 0.9 ml of 87% formic acid and 0.1ml of 30% hydrogen peroxide. After being left for thirty minutes at 25°C, the resulting solution was dried in vacuo, and hydrolysis was performed anaerobically as described previously.

3. Chromatography. Chromatography of the hydrolysate was performed on columns of Dowex 50-X8 according to the method of Moore and Stein[9]. A 0.9×100 cm column was used for the acidic and neutral amino acids and a 0.9×15 cm column for the basic amino acids. From the composition of the buffer solution, the detergent, BRIJ 35, was omitted throughout the experiments. With the samples of resin available, it was found that the rate of emergence of the amino acids was somewhat faster on the 100 cm column, although the order was identical with that given by Moore and Stein. Fractions (1ml) were collected on an automatic fraction collector equipped with a drop-counting device. The effluents were analyzed by the modified photoelectric ninhydrin procedure of Moore and Stein[10] by means of a Beckman Model DU spectrophotometer using 1cm cells. As a standard, L-leucine (Azinomoto Co.) was used to obtain the optical density-concentration curve, by which the optical density was found to be proportional to concentration. The values of color yields for amino acids relative to leucine given by Moore and Stein[10] were employed for calculating the yields of amino acids.

4. Spectroscopic determination of tryptophan. The tryptophan content was determined from the ultraviolet absorption spectrum of the intact apo-peroxidase in 0.1N NaOH according to the method of Goodwin and Morton[13]. In this determination, the extinctions at two wavelengths, 294.4 and 280 mμ, were employed and a correction for irrelevant continuous absorption was made with the use of extinctions at two wavelengths, i. e. 370 and 340 mμ.

---

[9] S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).
[10] S. Moore and W. H. Stein, *ibid.*, **211**, 907 (1957).
[11] E. C. Gjessing and J. B. Sumner, *Arch. Biochem.*, **1**, 1 (1943).
[12] E. Schram, S. Moore and E. J. Bigwood, *Biochem. J.*, **57**, 33 (1954).

EXPERIMENTAL RESULTS

A typical elution curve obtained from a seventy hour hydrolysate of apo-peroxidase on a 0.9×100 cm column of Dowex 50 is presented in Fig. 1. The elution curve for the basic amino acids and ammonia obtained from a twenty two hour hydrolysate of apo-peroxidase on a 0.9×15 cm column of Dowex 50 is presented in Fig. 2. In this curve, HEX represents hexosamine (probably glucosamine) as reported in the previous paper[2], and B will be a condensation product of true sugars and amino acids, because this effluent had a pale brown color.

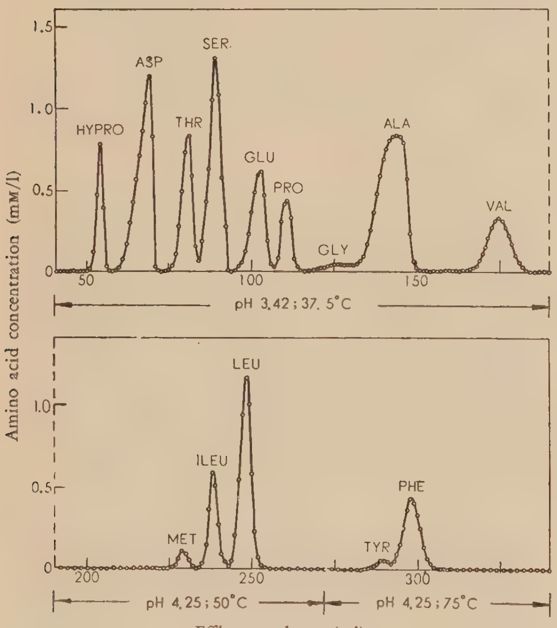


FIG. 1. Elution Curve for a 70 Hour Hydrolysate of 7.13 mg of Apo-peroxidase on a 0.9×100 cm Column of Dowex 50–X8.

The ninhydrin color yields have been corrected for baseline colors and for the different amino acids. The colors produced by proline and hydroxyproline were read at 440 mμ, and those by the other amino acids at 570 mμ. GLY=glycine, ALA=alanine, VAL=valine, MET=methionine, ILEU=isoleucine, LEU=leucine, TYR=tyrosine, PHE=phenylalanine, HYPRO=hydroxyproline, PRO=proline, THR=threonine, SER=serine, ASP=aspartic acid, and GLU=glutamic acid.

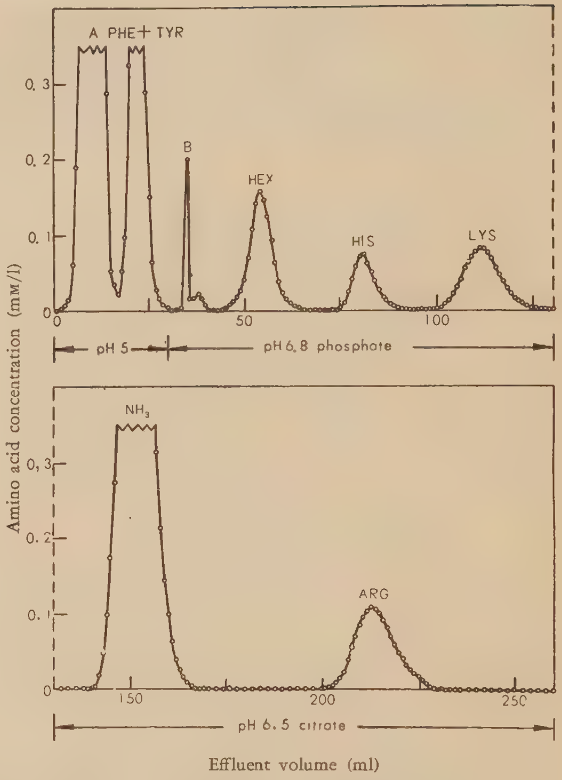


FIG. 2. Elution Curve for a 22 Hour Hydrolysate of 6.83 mg of Apo-peroxidase on a 0.9×15 cm Column of Dowex 50–X8 at 25°C.

The ninhydrin color yields have been corrected for baseline colors and for the different amino acids. The concentrations of A and B peaks are represented by the leucine equivalent. A=12 kinds of amino acids from HYPRO to LEU in Fig. 1, TYR=tyrosine, PHE=phenylalanine, B=unknown, HEX=hexosamine, HIS=histidine, LYS=lysine, and ARG=arginine.

The data of duplicate experiments after hydrolysis for twenty two and seventy hours are given in Table I. In the present experiments, several amino acids were lost during the course of prolonged hydrolysis even under

---

[13] T. W. Goodwin and R. A. Morton, *ibid.*, **40**, 628 (1946).

TABLE I. AMINO ACID RECOVERIES FROM APO-PEROXIDASE HYDROLYSATES

All the data are given as g of amino acid per 100 g of anhydrous protein.
The figures in parenthesis were not used in average or extrapolation.

Amino acid	Time of hydrolysis				Average or extrapolated value
	22 hours		70 hours		
Aspartic acid	12.33	12.65	12.40	12.57	12.49 ± 0.14*
Glutamic acid**	7.04	7.18	7.15	7.20	7.14 ± 0.07
Glycine	0.78	0.75	0.75	0.78	0.77 ± 0.02
Alanine	10.51	10.60	10.53	10.55	10.55 ± 0.04
Valine	(3.61)	(3.49)	4.44	4.25	4.35 ± 0.13
Leucine	8.72	8.56	8.83	8.76	8.72 ± 0.11
Isoleucine	(3.25)	(3.27)	3.75	3.56	3.66 ± 0.13
Serine	9.28	9.66	8.52	8.35	9.95† ± 0.17
Threonine	5.94	6.03	6.09	6.08	6.00 ± 0.10
Half cystine§	2.20	2.17	2.23	2.23	2.21 ± 0.02
Methionine**	0.97	1.02	0.93	1.00	0.98 ± 0.04
Proline	3.78	3.59	3.72	3.69	3.70 ± 0.08
Hydroxyproline	2.87	2.86	2.98	3.08	2.95 ± 0.10
Phenylalanine	5.52	5.63	5.56	5.60	5.58 ± 0.04
Tyrosine	1.11	1.08	1.06	1.06	1.08 ± 0.02
Histidine	1.16	1.18	1.07	1.12	1.20† ± 0.02
Lysine	1.79	1.78	1.80	1.84	1.80 ± 0.03
Arginine	3.39	3.50	3.35	3.37	3.40 ± 0.07
Ammonia	1.68	1.74	2.05	2.11	1.57† ± 0.04

* Average deviation.

** The values of methionine and glutamic acid include 4 and 3 per cent corrections for loss during chromatography[9].

† These values were obtained by extrapolating to zero time of hydrolysis in order to correct for apparent destruction or, in the case of amide groups, in order to correct for increased liberation of ammonia from the destruction of amino acids.

§ Determinations of cystine were performed as cysteic acid.

anaerobic condition. It must be noted that the recoveries of various amino acids during hydrolysis with 6 N HCl obtained by various authors have differed slightly[14~17]. These different values may be thought to have been caused according to the different conditions of hydrolysis, especially temperature, and the different kinds of sample. The recoveries of amino acids from Japanese-radish peroxidase a should differ from those of the other proteins hitherto investigated, because the former enzyme contained much carbohydrate. The authors, therefore, extrapolated the values to zero time of hydrolysis according to the suggestion made by Smith et al[15,16]. in order to correct for apparent destruction of these labile amino acids and, in the case of amide groups, in order to correct for increased liberation of ammonia from the destruction of amino acids. For the other stable amino acids, values were obtained by the average of four determinations. In addition, it is now generally recognized that many peptide bonds involving isoleucine and valine are difficult to hydrolyze[15,16,18,19]. As it is expected, the yields of valine and isoleucine increased upon hydrolysis for seventy hours, so the authors adopted these values. In such a manner, the values given

---

14) M. W. Rees, *ibid.*, **40**, 632 (1946).

15) E. L. Smith and A. Stockell, *J. Biol. Chem.*, **207**, 501 (1954).

16) E. L. Smith, A. Stockell and J. R. Kimmel, *ibid.*, **207**, 551 (1954).

17) C. H. W. Hirs, W. H. Stein and S. Moore, *ibid.*, **211**, 941 (1954).

18) E. J. Harfenist and L. C. Craig, *J. Am. Chem. Soc.*, **74**, 4216 (1952).

19) E. L. Smith, D. H. Spackman and W. Polglase, *J. Biol. Chem.*, **199**, 801 (1952).

in the last column of Table I were obtained. Cystine is unstable when hyrolysis is performed in the presence of carbohydrate and was hence determined as cysteic acid by the method of Schram et al.[12]. Tryptophan was determined independently by the spectrophotometric-method of Goodwin and Morton[13].

AMINO ACID COMPOSITION

The amino acid composition of Japanese-radish peroxidase a is summarized in Table II, together with its carbohydrate composition reported in the previous paper[2]. These values are computed from the data given in Table I with a correction for the protohematin content (1.166%).

The content of aliphatic hydroxy amino acids, i.e. serine, threonine and hydroxyproline, is very high, 90 residues of the total of 384 being estimated for the protein. The low contents of tyrosine, tryptophan, histidine, glycine and methionine are also noteworthy. The molecular weights of Japanese-radish peroxidase a were found to be 55700 and 54500 respectively when determined by means of osmometry and from the content of protohematin[1,6]. From the integral numbers of amino acid and carbohydrate residues and protohematin in the last column of Table II, a molecular weight of 51000 can be calculated. This value is some-

Table II. Amino Acid and Carbohydrate Composition of Japanese-Radish Peroxidase A

	Amino acid or carbohydrate per 100 g enzyme	Amino acid or carbohydrate residues per 100 g enzyme	N as per cent of total N	Calculated No. of residues for mol. wt. 54500	No. of residues to nearest integer
Aspartic acid	12.34	10.66	10.19	50.5	51
Glutamic acid	7.06	6.19	5.28	26.2	26
Glycine	0.76	0.58	1.12	5.55	6
Alanine	10.43	8.32	12.89	63.9	64
Valine	4.30	3.64	4.04	20.0	20
Leucine	8.62	7.44	7.24	35.9	36
Isoleucine	3.62	3.12	3.04	15.0	15
Serine	9.83	8.14	10.29	51.0	51
Threonine	5.93	5.03	5.48	27.1	27
Half cystine	2.18	1.85	1.99	9.88	10
Methionine	0.97	0.85	0.71	3.55	4
Proline	3.66	3.09	3.50	17.4	17
Hydroxyproline	2.92	2.52	2.45	12.2	12
Phenylalanine	5.51	4.91	3.67	18.2	18
Tyrosine	1.07	0.96	0.65	3.24	3
Tryptophan	0.92	0.84	0.99	2.46	2
Histidine	1.19	1.05	2.53	4.18	4
Lysine	1.78	1.56	2.68	6.64	7
Arginine	3.36	3.01	8.49	10.5	11
Amide NH_3	1.55*	1.46*	10.03	49.7*	50*
Total of amino acid	86.45	73.76	97.26	383.4	384
Hexosamine	4.97	4.42	2.83	15.0	15
Mannose	11.68	10.51		35.4	35
Xylose	3.10	2.73		11.3	11
Arabinose	1.04	0.93		3.80	4
Protohematin	1.17	1.17	0.81	1.00	1
Total	108.41	93.52	100.90	449.9	450

* These values are omitted from the total.

what lower than the former two values measured directly. This difference is interrelated to the somewhat low recovery of residues, though the recovery of nitrogen is reasonably satisfactory. This means that the recoveries of labile amino acids were not satisfactory, in spite that correction was performed by the extrapolating technique, or that an unknown component of a non-nitrogeneous matter other than the above-described constituents was contained in the enzyme molecule.

As a whole, the numbers of the non-amidized dicarboxylic acids and the net charge at pH 7 were computed at 25 and 7 respectively, each of which agreed fairly well with those values calculated from both titrimetric and electrophoretic experiments3).

Finally, it is obvious from the present experiment that Japanese-radish peroxidase a has a different composition from that of horseradish peroxidase II7). Especially, it is noteworthy that the former peroxidase contains hydroxyproline and tryptophan, while the latter contains neither of them8). Moreover, the basic amino acid compositions and the numbers of the non-amidized dicarboxylic acids of the two peroxidases differ from each other, so from these experimental results it can be well recognized that the isoelectric points of the two peroxidases are also different.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 1, p. 34~39, 1959]

Determination of Iodine Affinity of Starch by Amperometric Titration*

By Masaharu ITO and Shigekazu YOSHIDA

Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Tokyo

Received July 7, 1958

An amperometric modification of the potentiometric determination of starch-iodine affinity by Bates et al. has been developed. The procedure is essentially the same as that of the potentiometric method, but free iodine is determined amperometrically by using a rotating platinum electrode as an indicating electrode without the application of an external e.m.f. This method is of special advantage for routine work, since a sharp and exact end point is obtained. The amperometric titration curve can also easily be converted into the iodine affinity curve, which has been recommended as an exact method to evaluate the iodine-binding capacity of starchy materials. Using this technique, the iodine affinities of some starchy materials and their change during β-amylolysis are determined, successfully.

A potentiometric method, originally introduced by Bates, French and Rundle[1)] as a method for amylose determination, had been criticized by Schoch et at.[2)], but it is still an excellent method for the evaluation of iodine-binding capacity of starchy materials. By this method, however, it is fairly difficult to detect the end-point directly and, for evaluation of exact binding capacity, a plotting of bound iodine versus free iodine has been recommended[1~3)]. This curve, when coordinate and abscissa are interchanged, is very similar to the amperometric titration curve, which is the plot of free iodine versus added iodine and therefore, a sharp end-point may more easily be obtained by direct amperometric titration. Since no such treatise has so far been found in the literature, this article was undertaken from this point of view.

METHODS AND MATERIALS

The titration vessel was a 100-ml beaker equipped with a mantel into which water from the thermostat maintained at 25° was circulated. The rotating platinum electrode was of Kolthoff's A-type[4)] and was rotated at a speed of about 900 r.p.m. by a synchronous motor.

Titration was carried out essentially in the same way as in the potentiometric method derived by Bates et al. except that the total volume of the titrated solution was 50 ml. The solution was 0.05 N with respect to potassium iodide, 0.05 N to potassium chloride and contained an appropriate amount of starchy material requiring within 10 ml of the titer, which was 0.001 N with respect to iodine and 0.05 N to potassium iodide.

The starch sample was first defatted with methanol and dried. A weighed amount of the sample was well dispersed with 0.5 N potassium hydroxide, neutralized with hydrochloric acid and made to a definite volume. Dispersion of the sample was also effected by gentle heating after neutralization. In some experiments acetic acid was used for neutralization (pH 4.5), but the result was almost the same.

Fractionation of potato starch was made by Nikuni's modification[5)] of the method of Schoch et al. and A-fraction was twice recrystallized. Rice starch and its fraction were kindly furnished by Mr. H. Fukuba of the Ochanomizu Woman's University.

---

* Presented at the 176th Meeting of the Agricultural Chemical Society of Japan held in Tokyo, Sept. 28, 1957.

[1)] L. Bates, D. French and R. E. Rundle, *J. Am. Chem. Soc.*, **65**, 143 (1943).

[2)] S. Lansky, M. Kooi and T. J. Schoch, *ibid.*, **71**, 4066 (1949).

[3)] T. J. Schoch, in "Methods in Enzymology", Academic Press Inc., New York, 1957, Vol. 3, p. 13.

[4)] I. M. Kolthoff and J. J. Lingane, "Polarography", Interscience Publishers, New York, 1952, Vol. 1, p. 413.

[5)] Z. Nikuni and E. Fuwa, in "Standard Methods in Biochemical Experiments", (Egami et al. ed.) Bunkodo, Tokyo, 1953, p. 234.

RESULTS AND DISCUSSION

Current-voltage Curve of Free Iodine. According to Kolthoff[6], iodine can be determined amperometrically without applying an external e.m.f. using a saturated calomel electrode (S.C.E.) as a reference electrode. The current-voltage curve is shown in Fig. 1.

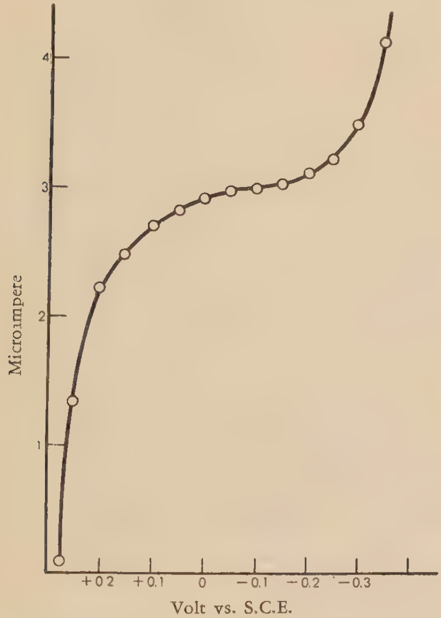


FIG. 1. Current-voltage Curve of Iodine at the Rotating Platinum Electrode.

After addition of 2.0 ml 0.001 N iodine to 50 ml solution containing 0.05 N KI, 0.05 N CH_3COOK and 0.05 N CH_3COOH

Change of pH between 3.6~5.6 in acetate buffer had no appreciable effect on the diffusion current.

Comparsion of Amperometric Titration Curve with Potentiometric Curve. In this experiments, free iodine in one titration medium was measured by two methods, namely amperometrically and potentiometrically. Though the end points were essentially the same, the amperometric end point was more easily detectable as shown in Fig. 2. Moreover, amperometric titration curve could easily be converted into the iodine affinity curve recommended by Bates et at.[1] and Schoch et al.[2,3]. The procedures are illustrated in Fig. 3 and are as follows:

1) Residual current is subtracted from each current reading. (solid line in Fig. 3)
2) Each point is calibrated for dilution caused by addition of the titer. (broken lines in Fig. 3)
3) Abscissa is changed from "added iodine" to "bound iodine" by subtracting from each point "free iodine" expressed as a reagent line. (chain line in Fig. 3)
4) Coordinate is changed from current to "free iodine".

In routine work, however, these procedures may not always be necessary.

In the amperometric method, the observed quantity is a linear function of free iodine, whereas, in the potentiometric method the relation is logarithmic. Accordingly, when it is necessary to compare the starch-iodine affinity over a wide range of iodine concentration, the potentiometric titration curve is of particular importance. However, in routine work, when only the stoichiometry is desired, amperometric titration is of special advantage, since a sharp end point is easily obtained as shown above, in this case the only essential instrument being a sensitive galvanometer.

On the other hand, the main disadvantage of this method consists in the variability of the electrode sensitivity. When many data have to be compared as titration curve as shown in Fig. 4, it is desirable to make a blank run per several titrations and calibrate each of them to eliminate this effects.

Titration of Several Whole Starches and Fractionated Starches. Amperometric titration curves of some commercial starches are shown in Fig. 4 and those of both A and B-fractions of potato and rice starch in Fig. 5. In these figures, each titration curve is calibrated for a small difference in electrode sensitivity and coordinate is expressed in free iodine rather than in current. Some disparity in iodine-

---

[6] I. M. Kolthoff and J. J. Lingane, "Polarography", Interscience Publishers, New York, 1952, Vol. 2, p. 946.

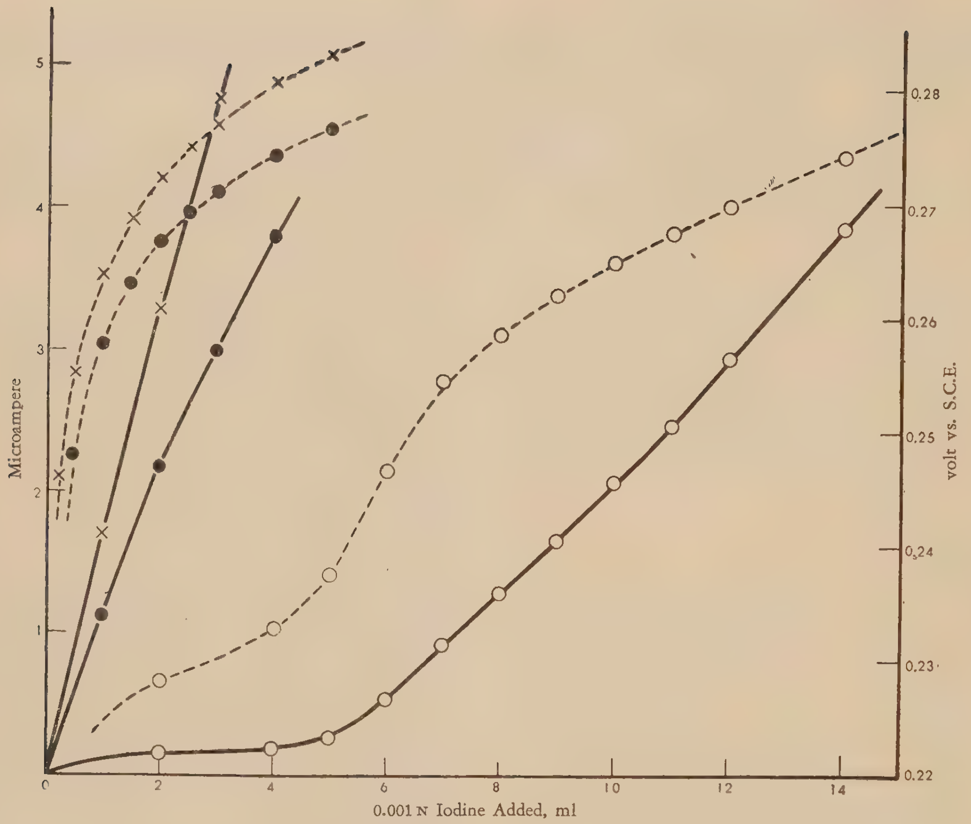


FIG. 2. Titration of Starch with 0.001 N Iodine

○ 20 mg potato starch (variety, Quebec)
● 20 mg waxy rice starch (variety, Saitama-mochi)
× reagent blank

The solid and broken lines indicate amperometric and potentiometric titration curves respectively. (not corrected for dilution) Acetic acid was used for neutralization. The applied e.m.f. was 0 volt vs. S.C.E. in amperometric titration. The value of 3.24% was obtained from this figure as per cent iodine affinity of potato starch.

binding capacity of starch sample is due to the difference of the variety of the plant species used. (e.g. potato starch in Fig. 2 and in Fig. 4) In potato and sweet potato starches, especially in the former, two phases are observed in iodine adsorption, thus the titration curve shows one more inflexion. Clearly, this feature results from B-fraction and it does not disappear at all in β-limit dextrin. (Fig. 5 and Fig. 6) From these reasons it is presumed that this may be due to a fairly long inner chain length of this type of starch. Attention was also paid to the low iodine-binding capacity of rice A-fraction as compared with potato A-fraction. (Fig. 5)

Change in Iodine-binding Capacity of Starch in the Course of β-Amylolysis. As another application of the above technique, both potato starch and potato A-fraction were analysed for iodine-binding capacity in the course of β-amylolysis. Thrice recrystallized sweet potato β-amylase was used throughout the trial. An appropriate amount of starchy material was dispersed with 0.5 N sodium hydroxide, neutra-

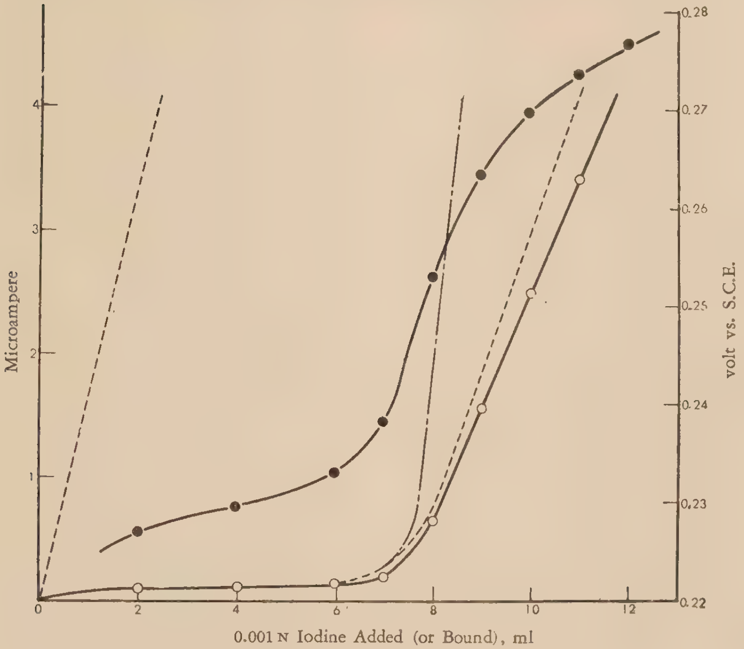


FIG. 3. Titration of Potato A-fraction with 0.001 N Iodine.

○ 5 mg potato A-fraction, amperometric titration
● 5 mg potato A-fraction, potentiometric titration

Experimental procedures were the same as in Fig. 2. The method to convert the titration curve (solid line) into the iodine affinity curve (chain line) is in text. The iodine affinity is obtained from the intersecting point of two straight lines in iodine affinity curve as recommended by Bates et al.. The value of 18.7% was obtained from this figure as per cent iodine affinity.

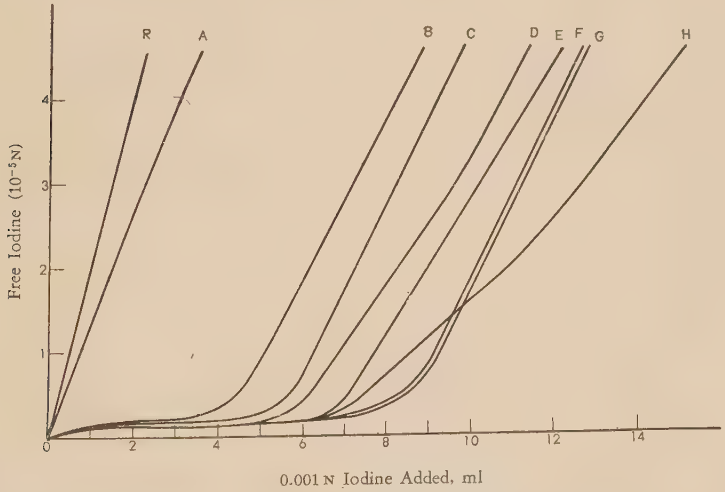


FIG. 4. Amperometric Titration of Some Whole Starches.

A waxy rice (Saitama-mochi)	B rice (Asahi)		C cassava	D sweet potato
E Kuzu (*Pueraria Iuirsuta, Matsum.*)	F wheat	G corn	H potato	R reagent blank

Each curve corresponds to 20 mg of starch and is calibrated for dilution. Hydrochloric acid was used for neutralization.

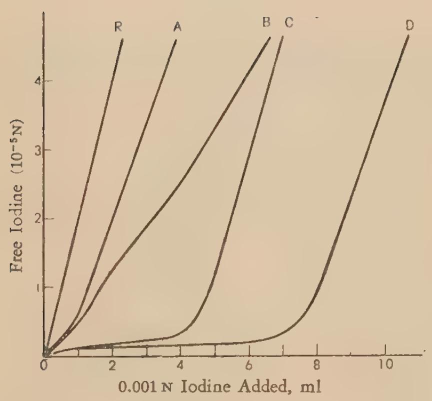


FIG. 5. Amperometric Titration of Fractionated Starches.

A 20 mg rice B-fraction
B 20 mg potato B-fraction
C 5 mg rice A-fraction
D 5 mg potato A-fraction
R reagent blank

The procedures were the same as in Fig. 4. The varieties of plant species were Asahi (rice) and Quebec (potato).

lized with 1 N acetic acid to pH 4.8 and digested with β-amylase at 30°. At definite time-intervals, each 5 ml aliquot was taken and added to 0.5 ml of 0.5 N sodium hydroxide to stop the enzyme action. From these samples, which corresponded to 4.04 mg of A-fraction

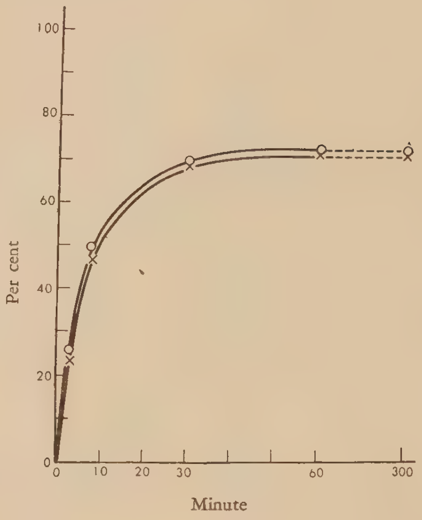


FIG. 7. Decrease in Iodine-binding Capacity of Potato A-fraction in the Course of β-Amylolysis

○ per cent hydrolysis
× per cent decrease in iodine-binding capacity

The procedures were almost the same as in Fig. 6 except that the solution of A-fraction was 0.5% and 2 ml portion was assayed for iodine affinity. See text.

and 8.08 mg of starch per ml, each 3 ml (A-fraction) and 1.5 ml (starch) portion was

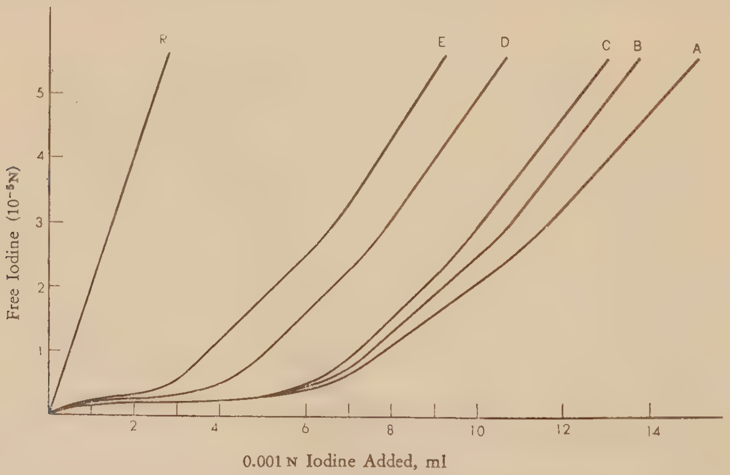


FIG. 6. Amperometric Titration of Potato Starch in the Course of β-Amylolysis.

A original B 1.5 minutes C 3 minutes D 8 minutes E 300 minutes R reagent blank

To 20 ml of 1% starch solution containing 0.05 M acetate buffer, pH 4.8, was added 1 ml of β-amylase solution (9 units per ml) in a total volume of 22.5 ml. After appropriate incubation time each 5 ml aliquot was added to 0.5 ml of 0.5 N sodium hydroxide and from these samples 3 ml portion, corresponding to 24.2 mg of the original starch, was assayed for iodine affinity amperometrically.

analysed for reducing sugar by Schwimmer's method⁽⁷⁾ and 2 ml (A-fraction) and 3 ml (starch) portion for iodine-binding capacity. Typical results are shown in Figs. 6, 7 and 8.

As reported by Peat et al.⁽⁸⁾ and other workers⁽⁹,¹⁰⁾, it was confirmed that potato amylose is hydrolysed to only approximately 70 per cent of the theoretical maximum by crystalline sweet potato β-amylase. In Fig. 7, it is shown that the per cent decrease in iodine-binding capacity (corresponding to the same amount of the original material) is almost equal to the per cent hydrolysis at every stage of β-amylolysis. This suggests that the change in iodine-binding capacity of residual amylose itself after β-amylolysis is very small, even if any change occurs. In the case of β-amylolysis of starch, the hydrolysis limit of amylose seems to be still lower, since, as seen in Fig. 8, the decrease in iodine-binding capacity amounted to only 60 per cent of the original. (The decrease in iodine-binding capacity in Fig. 8 is considered to be almost equal to the hydrolysis per cent of the amylose component as shown in Fig. 7) In this case, a lag phase of the decrease in iodine-binding capacity is observed in the initial stage of hydrolysis. This clearly indicates the preferential hydrolysis of the amylopectin component in this stage as the result of the difference in affinity of the two starch components to β-amylase⁽¹¹⁾.

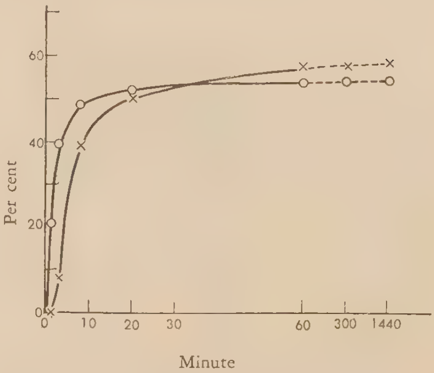


FIG. 8. Decrease in Iodine-binding Capacity of Potato Starch in the Course of β-Amylolysis

○ per cent hydrolysis
× per cent decrease in iodine-binding capacity
The procedures were the same as in Fig. 6.

Acknowledgement. The authors wish to express their sincere thanks to Prof. Z. Nikuni of Osaka University for his valuable suggestions. Thanks are also due to Mr. S. Suzuki, Food Research Institute, Ministry of Agriculture and Forestry, and to Mr. H. Fukuba, Ochanomizu Woman's University, for kindly supplying the starch samples.

Addendum. While this paper was in press, it was pointed out to the authors by Dr. T. Kobayashi, University of Tokyo, that the similar investigation had already appeared. Namely, B. L. Larson et al.⁽¹²⁾ described an amperometric adaptation of the dead-stop titration for determining the iodine-binding capacity of starch.

---

7) S. Schwimmer, *Cereal Chem.*, **24**, 167 (1947).

8) S. Peat, S. J. Part and W. J. Whelan, *J. Chem. Soc.*, **1952**, 705.

9) E. F. Neufeld and W. Z. Hassid, *Arch. Biochem. Biophys.*, **59**, 405 (1955).

10) A. Baba and H. Kojima, *J. Agr. Chem. Soc. Japan*, **28**, 598 (1954), **28**, 603 (1954).

11) R. H. Hopkins and B. K. Jha, *Biochem. J.*, **46**, 319 (1950).

12) B. L. Larson, K. A. Cilles and R. Jenness, *Anal. Chem.*, **25**, 802 (1953).

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 1, p. 40~45, 1959]

Polarographic Studies on Sarkomycin

Part I. Polarography of Sarkomycin, its Degradation or Polymerization Products, and 3-Methyl-2-oxocyclopentane-1-carboxylic Acid

By Keiji Ida

Meiji Seika Kaisha, Ltd.

Received July 8, 1958

Polarogram of sarkomycin (3-methylene-2-oxocyclopentane-1-carboxylic acid), isolated by the countercurrent distribution method, gives two reduction waves in the acid region (-0.89 v. and -1.14 v. vs. S.C.E. at pH 1.7) and one wave in the neutral and the alkaline regions (-1.44 v. vs. S.C.E. at pH 8.3). Analysis of the slope of each wave at pH 1.7 gives values of 0.063 v. and 0.076 v. Other biologically inactive fractions show one wave (-1.18 v. vs. S.C.E. at pH 1.5), only in the acid region. Crystals D and Z, relatives of sarkomycin, show a wave in the acid region. 3-Methyl-2-oxocyclopentane-1-carboxylic acid shows one wave below pH 4.0. Calibration curves from reducing currents and sarkomycin concentrations are linear, only in the same sample. But the decreasing rate of the reducing current is not proportional to that of biological activity of sarkomycin after storage. The method is, therefore, not applicable for the determination of sarkomycin without modification.

Sarkomycin is an antitumor antibiotic, isolated from the fermentation liquor of Streptomyces erythrochromogenes by H. Umezawa et al.[1,2]. The chemical structure of the main effective component was determined by I. R. Hooper et al. and proved to be 3-methylene-2-oxocyclopentane-1-carboxylic acid[3]. Later, Y. Matsuda et al. reported on the polarography of sarkomycin that sarkomycin gave only one reduction wave and was assayed by the polarographic method directly[4]. But these findings are quite different from the author's results, attained by polarography since the beginning of the test-production of sarkomycin; sarkomycin has two reduction waves which in the alkaline media appear to be one wave on account of the approach of their half-wave potentials. 3-Methyl-2-oxocyclopentane-1-carboxylic acid also, shows a wave in the acid media.

EXPERIMENTAL

Apparatus: In early work a "Yanagimoto" Model PS-51 polarograph was employed to obtain polarograms of sarkomycin. Later, a "Yanagimoto" Model PR-1 polarorecorder was connected with the above Model PS-51. Alternating current polarography was carried out by using a "Yanagimoto" Model PA-101 Polarograph. The used cell was a H-type cell equipped with an agar bridge to a saturated calomel electrode to determine the half-wave potentials. The dropping mercury electrode has a value of $m^{2/3}t^{1/6}$ of 1.71 $mg^{2/3}$ $sec^{1/6}$. The temperature was maintained constant at 21°C during electrolysis.

Samples:

Sarkomycin acid oil, neutral extract of sarkomycin, and amorphous powder of sarkomycin sodium salt. Samples were prepared as described by H. Umezawa et al.[1,2]. The filtered fermentation

---

[1] H. Umezawa, T. Takeuchi, K. Nitta, Y. Okami, T. Yamamoto and S. Yamaoka, *J. Antibiotics* (Japan), (A) **6**, 147-52 (1953).

[2] H. Umezawa, T. Yamamoto, T. Takeuchi, T. Osato, Y. Okami, S. Yamaoka, T. Okuda, K. Nitta, K. Yagishita, R. Utahara and S. Umezawa, *Antibiotics and Chemotherapy*, **4**, 514-20 (1954).

[3] I. R. Hooper, L. C. Cheney, M. J. Cron, O. B. Fardig, D. A. Johnson, D. L. Johnson, F. M. Palermiti, H. Schmitz and W. B. Wheatley, *Antibiotics and Chemotherapy*, **5**, 585-95 (1955).

[4] Y. Matsuda and M. Matsuzaki, *Yakugaku Kenkyu*, **29**, 520-8 (1957).

liquor was extracted with ethyl acetate at pH 2.0 and the active component in the solvent phase was purified by repeated extraction into water at pH 7.0 and back-extraction into ethyl acetate at pH 2.0. Sarkomycin acid oil was obtained by concentrating the final solvent layer in vacuo, and neutral extract of sarkomycin by re-extraction of the solvent layer into water at pH 7.0. Amorphous powder of sarkomycin sodium salt was prepared by lyophilizing the neutral extract of sarkomycin in vacuo.

Sarkomycin calcium salt. Sarkomycin calcium salt was prepared as described by T. Hara et al[5]. Butyl acetate extract of sarkomycin was fractionally neutralized with an aqueous suspension of calcium salt carbonate. Sarkomycin calcium was precipitated by the addition of ethanol to the main fraction, followed by vacuum-drying.

3-Methyl-2-oxocyclopentane-1-carboxylic acid. A sample, synthesized by T. Hara et al.[6], was used.

Crystal Z. Crystal Z as reported by T. Hara et al.[5] was used. When sarkomycin acid oil was stored in a refrigerator for a long time, a large amount of crystals appeared. Recrystalization was carried out from water.

Crystal D. Crystal D, also reported by T. Hara et al.[5], was used. When calcium salt of crystal Z was allowed to be kept in an aqueous solution at room temperature, calcium salt of crystal D precipitated. Crystal D also appeared when the aqueous solution of calcium salt of sarkomycin was stored at room temperature.

Polarography:

Polarography of the fractions of sarkomycin by countercurrent distribution method. An aqueous solution of sarkomycin sodium salt (300 units/ml.) was subjected to 60 transfer-countercurrent distribution, using ethyl acetate and phosphate buffer solution (pH 4.8).

One-tenth ml. of each aqueous fraction was biologically assayed by the paper-disc method. On the other hand, pH of the aqueous phase was brought up to 2.0 with sulfuric acid, followed by extraction into the ethyl acetate phase. Three ml. of ethyl acetate phase was concentrated in vacuo to the residue, which was weighed and then dissolved in 3 ml. of 0.1M citric acid to be submitted to polarography. Fig. 1 shows the results.

Polarography of the substance from transfer 17. Three ml. of the ethyl acetate phase of transfer 17, which was one of the most biologically active and most weighty fractions, was concentrated in vacuo and the residue (3.45 mg.) was dissolved in 3 ml. of the acid solution of the Britton-Robinson buffer solution, and polarographed adjusting the pH with N sodium hydroxide or N hydrochloric acid. Polarograms and the correlation between pH and half-wave potentials are shown in Fig. 2 and Table I. The current as shown in Table II was not kinetic. Analysis of the slope of the first and the second waves at pH 1.7 gave values of 0.063 v. and 0.076 v., respectively. This

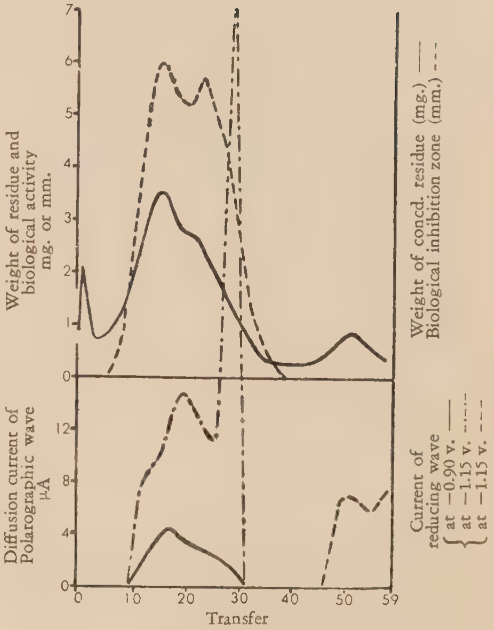


FIG. 1. Analysis of Fractions by Countercurrent Distribution of Sarkomycin.

TABLE I. HALF-WAVE POTENTIAL OF SARKOMYCIN AT VARIOUS pH

pH	Half-wave potential (vs. S.C.E.)	
	I	II
1.7	−0.89 v.	−1.14 v.
2.2	−0.93	−1.15
3.0	−0.99	−1.19
3.9	−1.06	−1.23
5.0	−1.15	−1.27
6.0	−1.22	−1.30
7.2	−1.35 v.	
8.3	−1.44	

---

[5] T. Hara, H. Yamada, K. Ida and Y. Yamada, *J. Antibiotics* (Japan), (B) **9**, 184-8 (1956).

[6] T. Hara, Y. Yamada and E. Akita, *J. Antibiotics* (Japan), (A) **10**, 80-1 (1957).

fraction is considered to be the pure sarkomycin, thus the polarograms may be said to be those of pure sarkomycin.

TABLE II. CORRELATION BETWEEN REDUCING CURRENT OF SARKOMYCIN AND HEIGHT OF Hg-pool

Hg-height (h)	Current (Id)	Id/h$^{\frac{1}{2}}$
81 cm	I 4.59 μA	0.51
	II 9.6	1.07
64	I 4.27	0.53
	II 8.92	1.12
49	I 3.97	0.57
	II 8.29	1.18

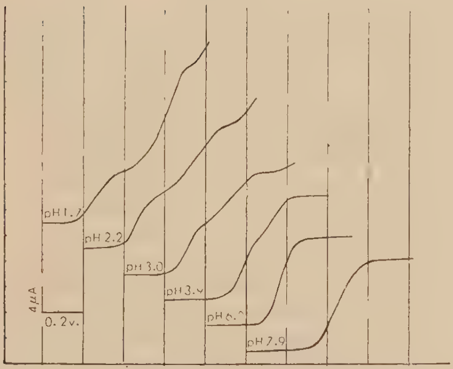


FIG. 2. Polarograms of Sarkomycin from −0.7 v. vs. S.C.E. (Transfer 17).

Polarography of the substance from transfer 55. The concentrated residue of 3 ml. of ethyl acetate phase of transfer 55 (0.50 mg.), which was one of the biologically inactive and polarographically reducible fractions, was dissolved in 3 ml. of 0.1M citric acid, followed by polarography adjusting the pH with saturated aqueous solution of dibasic sodium phosphate or N hydrochloric acid. The half-wave potentials and correlation between the current and height of mercury pool are shown in Table III and the polarograms in Fig. 3. As shown in the Table III, the current is also not kinetic.

Polarography of sarkomycin acid oil. Sarkomycin acid oil was dissolved with a sodium bicarbonate aqueous solution. The resulting solution was diluted with McIlvaine and Britton-Robinson buffer solutions and polarographed. The waves and the half-wave potentials were identical with the results obtained for sarkomycin by the countercurrent distribution method.

TABLE III. HALF-WAVE POTENTIAL OF THE SUBSTANCE FROM TRANSFER 55 AT VARIOUS pH AND CORRELATION BETWEEN CURRENT AND HEIGHT OF Hg-pool

pH	Half-wave potential (vs. S.C.E.)	Hg-height (h)	Current (Id)	Id/h$^{\frac{1}{2}}$
1.5	−1.18 v.	81 cm		
2.2	−1.25	81		
3.0	unrecognizable	81		
1.5		81	70.0 μA	9.71
1.5		64	68.5	8.56
1.5		49	68.0	7.77

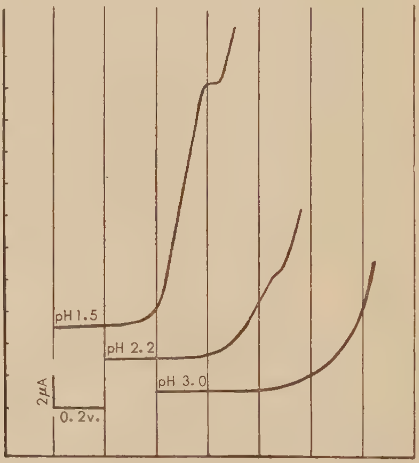


FIG. 3. Polarograms of Substance in Transfer 55 from −0.7 v. vs. S.C.E.

Polarography of neutral extract of sarkomycin. The neutral extract of sarkomycin was diluted with McIlvaine and Britton-Robinson buffer solutions and polarographed. The results were identical with sarkomycin obtained by the countercurrent distribution method.

Polarography of amorphous powder of sarkomycin sodium salt. Most samples of amorphous powder give two reduction waves of sarkomycin as well as another wave, derived probably from a relative induced by freeze-drying. The latter half-wave potentials are more positive than those of sarkomycin (−0.76 v. vs. S.C.E. at pH 2.0, −0.94 v. vs. S.C.E. at pH 4.0 and −1.12 v. vs. S.C.E. at pH 6.0). In any case, the current of the first wave of sarkomycin is smaller and that of the second is greater than the respective current of sarkomycin by the countercurrent distribution method, and the ratio of both currents at pH 2.0 (the second/the first) reached to 7.88 in a certain sample (the ratio of sarkomycin by countercurrent distribution method: 2.09).

Polarography of sarkomycin calcium salt. Each fraction of sarkomycin calcium salt having biological activity, showed one reduction wave which appeared to be comprised in one wave on account of the approach of both waves.

Polarography of crystal Z. Fifty mg. of crystal Z was dissolved in 10 ml. of water. Half a ml. of the resulting solution was diluted to 5 ml. with McIlvaine buffer solution and 0.1N hydrochloric acid and polarographed. The data and polarograms are given in Table IV and Fig. 4.

TABLE IV. HALF-WAVE POTENTIAL OF CRYSTAL Z AT VARIOUS pH AND CORRELATION BETWEEN CURRENT AND HEIGHT OF Hg-pool

pH	Half-wave potential (vs. S.C.E.)	Hg-height (h)	Current (Id)	$Id/h^{\frac{1}{2}}$
1.2	−1.18 v.	81 cm		
1.5	−1.19	81		
1.7	−1.20	81		
2.0	−1.22	81		
2.1	−1.23	81		
3.0	−1.27	81		
4.0	unrecognizable	81		
1.2		81	9.0 μA	1.0
1.2		64	8.8	1.1
1.2		49	8.8	1.26

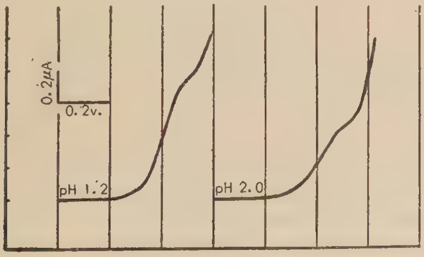


FIG. 4. Polarograms of Crystal Z from −0.8 v. S.C.E.

TABLE V. HALF-WAVE POTENTIAL OF CRYSTAL D AT VARIOUS pH AND CORRELATION BETWEEN CURRENT AND HEIGHT OF Hg-pool

pH	Half-wave potential (vs. S.C.E.)		Hg-height (h)	Current (Id)	$Id/h^{\frac{1}{2}}$
2.4	−1.06 v.	−1.24 v.	81 cm		
3.0	−1.08	—	81		
3.8	−1.10	—	81		
4.5	−1.15	—	81		
6.0	unrecognizable		81		
2.4			81	I 0.28 μA	0.031
				II 2.08	0.23
2.4			64	I 0.28	0.035
				II 2.16	0.27
2.4			49	I 0.28	0.041
				II 2.24	0.32

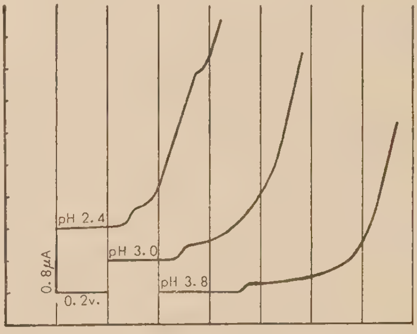


FIG. 5. Polarograms of Crystal D from −0.8 v. vs. S.C.E.

Polarography of crystal D. Polarography of crystal D was conducted according to the same procedure as crystal Z, using 50 mg. of the sample. The results are shown in Table V and Fig. 5.

Polarography of 3-methyl-2-oxocyclopentane-1-carboxylic acid. Fifty mg. of 3-methyl-2-oxocyclopentane-1-carboxylic acid was dissolved in 5 ml. of water. Half a ml. of the resulting solution was diluted to 10 ml. with McIlvaine buffer solution and polarographed. The data and polarograms are given in Table VI and Fig. 6.

Correlation between reducing currents and sample concentrations. One hundred and fifty mg. of sarkomycin sodium salt (Lot No. RSK 27; 1.5 units/mg.) was dissolved in 20 ml. of water and 0.5, 1, 1.5, 2, and 3 ml. of the solution was diluted to

TABLE VI. HALF-WAVE POTENTIAL OF 3-METHYL-2-OXOCYCLOPENTANE-1-CARBOXYLIC ACID

pH	Half-wave potential
2.3	−1.24 v. vs. S.C.E.
3.0	−1.27
4.0	−1.31
5.0	unrecognizable

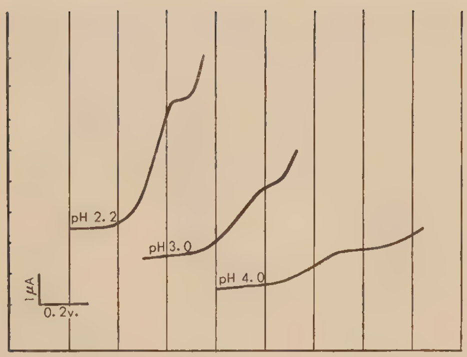


FIG. 6. Polarograms of 3-Methyl-2-oxocyclopentane-1-carboxylic Acid from −0.9 v. vs. S.C.E.

TABLE VII. STABILITY OF REDUCING CURRENT AND BIOLOGICAL ACTIVITY AFTER 24 AND 48 HOURS

Temperature	pH	Initial		After 24 hrs.		After 48 hrs.	
20°C	2	7.4 μA	12 units/ml	7.2 μA	12 units/ml	7.2 μA	12 units/ml
	4	7.7	18	7.2	16	7.0	16
	7	7.4	12	7.4	12	7.3	10
0°C	2	7.4	12	7.4	13	7.4	13
	4	7.7	18	7.4	19	7.1	16
	7	7.4	12	7.4	15	7.1	10

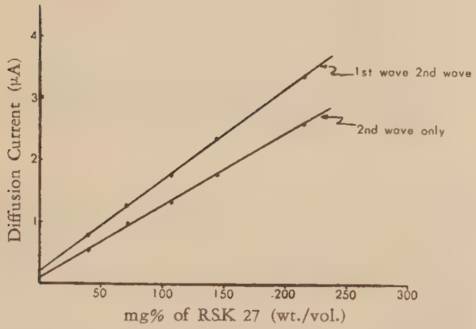


FIG. 7. Calibration Curves for Sarkomycin Sodium Lot No. RSK 27 (1.5 units/mg.)

10 ml. with McIlvaine buffer solution (pH 4.0) and polarographed. The calibration curves were linear as they are seen in Fig. 7. The diluted solution, left standing at pH 2, 4, and 7 at 20°C for 48 hours, showed no decrease of reducing current and biological activity (Table VII). However the aqueous solution of sarkomycin sodium salt (pH 6.0, 360 units/ml.), left standing at 0° and 15°C, lost biological activities, out of proportion to the decrease in the reducing currents (Table VIII).

Alternating current polarography of sarkomycin. Half a ml. of aqueous solution of sarkomycin (12 units/ml.) was diluted to 50 ml. with McIlvaine buffer solution (pH 2, 4, and 7). The polarograms are shown in Fig. 8. The obtained mhos of the first waves were proportional to concentrations of the same sarkomycin sample.

DISCUSSION

Sarkomycin was investigated to be 3-methylene-2-oxocyclopentane-1-carboxylic acid by I. R. Hooper et al., but it is not obtained in a crystal form. The author's polarographic investigations were, therefore, carried out with sarkomycin, isolated by means of the counter-current distribution method. The results are quite different from Matsuda's data; sarkomycin shows two reduction waves below pH 7, both waves appearing to be one wave over pH 7. Alternating current polarography also approves two waves of sarkomycin. Analysis of the slope of each wave at pH 1.7 gives values of 0.063 v. and 0.076 v., respectively

TABLE VIII. EFFECT ON REDUCING CURRENT AND BIOLOGICAL ACTIVITY OF SARKOMYCIN SODIUM AQUEOUS SOLUTION BY STORAGE

Temperature	pH	Initial			After 1 week		
		Current (C)	Potency (P)	C/P	Current (C)	Potency (P)	C/P
0°C	6.0	7.4 × 3000 μA	360 unit/ml	61.6	6.0 × 3000 μA	200 unit/ml	90
20°C	6.0	4 × 3000	360	61.6	2.7 × 3000	45	180

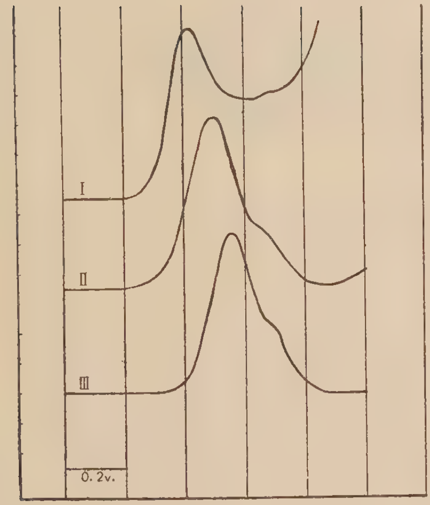


FIG. 8. AC-polarograms of Sarkomycin from −0.7 v. vs. S.C.E. I, pH 2.0, 4 μmho/mm.; II, pH 3.8, 8 μmho/mm.; III, pH 6.8, 4 μmho/mm.

While sarkomycin is kept in an aqueous solution, both waves at pH 2, which are well defined from each other, approach and appear to be only one wave.

3-Methyl-2-oxocyclopentane-1-carboxylic acid gives one polarographic wave below pH 4.0. Crystals Z and D, which are considered as degradation or polymerization products, give one polarographic wave only in the acid region. Their half-wave potentials are similar to those of the second wave of sarkomycin.

The kinetic current which proves the reaction rate is not recognized in sarkomycin and its relatives.

Sarkomycin has a possibility to be reduced at methylene and carbonyl radicals. At present, this is not cleared, but future research will make its reduction mechanism clear.

The polarography of sarkomycin has no availability for determination without pretreatment, because it is indicated that the correlation between reducing currents and concentrations of one sample is not entirely applicable to the other sample.

SUMMARY

1. Polarography of each fraction isolated from sarkomycin by the countercurrent distribution method revealed the existence of two components, showing polarographic waves. One of them shows biological activity, while the other shows no biological activity.

2. The pure sarkomycin obtained by the countercurrent distribution method, showed two reduction waves in the acid region and one wave in neutral and alkaline regions, resulting in the approach of both waves.

3. Analysis of the slope of the reducing waves of sarkomycin gives values of 0.063 v. (for the first wave) and 0.076 v. (for the second).

4. Crystals Z and D, which are considered as degradation or polymerization products, were polarographed and showed one polarographic wave in the acid region.

5. 3-Methyl-2-oxocyclopentane-1-carboxylic acid was polarographed and showed one wave below pH 4.0.

6. The correlation between reducing currents and concentrations of sarkomycin solution is linear, restrictive to the same sample. However, polarographic method can not be applied for determination, because the decrease rate of biological activity is not proportional to that of the reducing current.

Acknowledgement. The author wishes to acknowledge the kind guidance and valuable suggestion extended by Dr. Isamu Tachi, Professor of Kyoto University and also to express appreciation to Mr. Kametaro Urashima, President of Meiji Seika Kaisha, Ltd. for his encouragement throughout this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 1, p. 46~48, 1959]

A New Basic Triple Salt Containing Magnesium Hydroxide

Part II. The Quaternary System KCl-$MgCl_2$-$Mg(OH)_2$-H_2O at 100°

By Michio NAKAYAMA

Central Research Institute, Japan Monopoly Corporation

Received July 9, 1958

The quaternary system KCl–$MgCl_2$–$Mg(OH)_2$–H_2O and the associated systems, (a) KCl–$Mg(OH)_2$–H_2O and (b) $MgCl_2$–$Mg(OH)_2$–H_2O were investigated at 100°. Though the isotherm (a) exhibited nothing new, isotherm (b) exhibited a new basic magnesium chloride, $MgCl_2 \cdot 2Mg(OH)_2 \cdot 6H_2O$, as a solid phase in such a solution having a higher concentration of magnesium chloride than 51.5 gram per 100 gram water. The solid phases of quaternary isotherm were the new basic magnesium chloride, bischofite, carnallite, potassium chloride and magnesium hydroxide.

INTRODUCTION

In the previous paper[1], the 100° isotherm of systems, K_2SO_4–$Mg(OH)_2$–H_2O, $MgSO_4$–$Mg(OH)_2$–H_2O and K_2SO_4–$MgSO_4$–$Mg(OH)_2$–H_2O were reported. Following this report, the quaternary system KCl–$MgCl_2$–$Mg(OH)_2$–H_2O and the associated systems KCl–$Mg(OH)_2$–H_2O and $MgCl_2$–$Mg(OH)_2$–H_2O were investigated at 100°, in this study. From any system investigated in this study, the new basic triple salt (the author has named this "NS salt B" after the initials of its discoverers) did not appear, but a new basic magnesium chloride was obtained from the three component system $MgCl_2$–$Mg(OH)_2$–H_2O. The chemical formula of this basic magnesium chloride was decided to be $MgCl_2 \cdot 2Mg(OH)_2 \cdot 6H_2O$.

The 100° isotherm of the quinary system KCl–$MgCl_2$–K_2SO_4–$MgSO_4$–$Mg(OH)_2$–H_2O will be published in a forthcoming paper.

EXPERIMENTAL

Definite amounts of purity-tested potassium chloride, magnesium chloride, magnesium hydroxide and water were placed in Erlenmeyer flasks. The flasks were then immersed in an oil thermostat and sealed with rubber plugs after they had attained the desired temperature. The liquid temperature was maintained at 100°±0.5°. Instead of stirring the liquid, the flasks were immersed in the thermostat for a sufficient period (100~500 hours) and rotated from time to time by hand.

Sampling A pipette whose tip was cut off and covered with small cotton and gauze as a filtering medium having been previously heated at the same temperature was inserted into the solution, attention being paid not to disturb the solid residue, and a liquid phase sample was obtained by the use of a "pipetter" the pipetting apparatus. About 15 gram of the solution was usually taken for analysis.

Method of analysis Liquid phase samples were analyzed for potassium, magesium, hydroxide and chloride.

(a) **Potassium**, (b) **Magnesium**, and (c) **Hydroxide** The same method as described in the previous paper was adopted.

(d) **Chloride** The method of K. Fajans[2] was adopted, if necessary, after the sample was neutralized with N/10 sulfuric acid.

Solid phase samples were not analyzed chemically but examined by both the powder X-ray diffraction and the petrographic microscope.

RESULTS AND DISCUSSION

System KCl–$Mg(HO)_2$–H_2O at 100° To date, no reports have yet appeared concerning this system. Results obtained from this system are shown in Fig. 1. In this system, no double salt formation took place and nothing new resulted.

---

[1] J. Sugi and M. Nakayama, This Bulletin, **22**, 208, (1958).

[2] I. M. Kolthoff and V. A. Stenger, "Volmetric Analysis" Vol. 1, 2nd. Ed., 1947, p. 245.

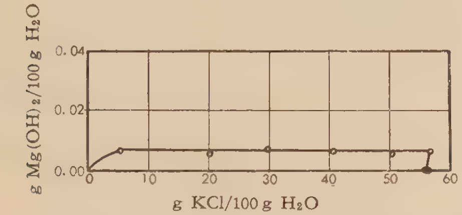


FIG. 1. The 100° Isotherm of the System $KCl-Mg(OH)_2-H_2O$.

System $MgCl_2-Mg(OH)_2-H_2O$ at 100° Nothing has been reported on this system at 100°, but at other temperatures some reports such as those of T. Maeda and S. Yamane[3] at 25° and 50°, J. D'Ans and others[4] at 25° and O. Lahrmann[5] at 70° have been published, that is, $MgCl_2 \cdot 3Mg(OH)_2 \cdot 8H_2O$ crystallized at 25° and 50°, or $MgCl_2 \cdot 3Mg(OH)_2 \cdot 4H_2O$ crystallized at 70° in this system. Moreover, according to the report of J. D'Ans, the latter was produced by heating the former crystal at 110° in the air. Conjecturing the depositing crystal in this system at 100° according to these reports, it is considered proper to crystallize $MgCl_2 \cdot 3Mg(OH)_2 \cdot 4H_2O$.

Results obtained from this system, however, showed a new basic magnesium chloride. The chemical formula of this double salt was decided to be $MgCl_2 \cdot 2Mg(OH)_2 \cdot 6H_2O$.

Analytical data are:

found: Mg, 22.70; OH, 20.57; Cl, 22.87; H_2O, 33.86%,

calcd. for $Mg_3(OH)_4Cl_2 \cdot 6H_2O$: Mg, 22.80; OH, 21.26; Cl, 22.16; H_2O, 33.78%.

The confirmation of the existence of the new basic double salt was also made by the powder X-ray diffraction method.

Results obtained from this system are shown in Fig. 2. The new basic double salt crystallized in such a solution that the concentration of magnesium chloride was higher than 51.5 gram per 100 gram water. In a solution in which the concentration of magnesium chloride was lower than 51.5 gram per 100 gram water, the solid phase was magesium hydroxide. The solubility of magnesium hydroxide was quite large in comparison with those of other systems in the series of this study.

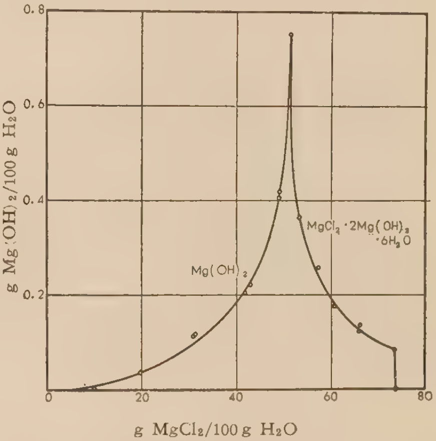


FIG. 2. The 100° Isotherm of the System $MgCl_2-Mg(OH)_2-H_2O$.

System $KCl-MgCl_2-Mg(OH)_2-H_2O$ at 100° Nothing has yet been reported on this quaternary system, but concerning the ternary system $KCl-MgCl_2-H_2O$ at 100°, two investigations were made by N. S. Kurnakov and others[6] in 1932 and A. N. Campbell and others[7] in 1934.

Results obtained from this system are shown in Fig. 3, but the concentration of the dissolved magnesium hydroxide was so small that the diagram must be divided into two, namely, those of potassium chloride-magnesium chloride and magnesium chloride-magnesium hydroxide. The solubility of magnesium hydroxide in

---

3) T. Maeda and S. Yamane, *Bull. Inst. Phys. Chem. Res.*, **1**, 31, (1928).

4) J. D'Ans, W. Busse and H. E. Freund, *Kali und Steinsalz*, **1955**, H. 8, 3.

5) O. Lahrmann, *Dissert München T. H.*, **1910**, s. 20.

6) N. S. Kurnakov, D. P. Manoev and N. A. Osokoreva, *Kalij* [*russ*], **1932**, Nr. 2, p. 25.

7) A. N. Campbell, K. W. Downes and C. S. Samis, *J.A.C.S.*, **56**, 2507, (1934).

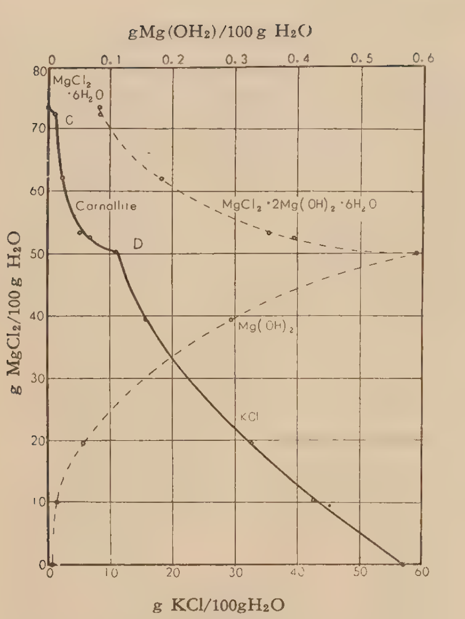


FIG. 3. The 100° Isotherm of the System KCl–$MgCl_2$–$Mg(OH)_2$–H_2O.

this system, discussing interrelation with the concentration of magnesium chloride, as shown by the broken-line in Fig. 3, was closely analogous to those of system $MgCl_2$–$Mg(OH)_2$–H_2O. The solid phases of this system were bischofite, carnallite, basic magnesium chloride ($MgCl_2 \cdot 2Mg(OH)_2 \cdot 6H_2O$), potassium chloride and magnesium hydroxide.

Compositions of the liquid phase at invariant points, C and E are

point C: KCl, 1.10; $MgCl_2$, 72.49; $Mg(OH)_2$, 0.087 g/100 g H_2O
point E: KCl, 10.89; $MgCl_2$, 50.18; $Mg(OH)_2$, 0.592 g/100 g H_2O

These compositions excluding magnesium hydroxide are in close agreement with those obtained by N. S. Kurnakov and others.

Acknowledgement The author's grateful thanks are due to Prof. Dr. Jiro Sugi and Prof. Dr. Shumpei Oka of Tokyo University for their kind advice and encouragement throughout the coure of this study. Indebts are also due to B. Agr. T. Matsuo and Miss S. Takabayashi for their assistance in carrying out this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 1, p. 49~59, 1959]

Studies on the Protease of *Pseudomonas*

Part IV. Factors Affecting the Enzyme Production, Especially on the Effect of Calcium

By Kazuyuki Morihara

Research Laboratory of Shionogi & Co., Ltd., Amagasaki, Hyogo

Received July 17, 1958

The extracellular proteinase of *Ps. myxogenes* sp. was found to be produced by cell suspension in a phosphate buffer of pH. 7.0 containing a carbon source and calcium ion under aerobic conditions.

Various factors having affect on the proteinase production were studied, and calcium was found to be essential in its production. However, in cells prepared from a synthetic medium without calcium, the enzyme producing ability was possessed similarly as in those from the medium containing calcium, and the production was not altered by amino acid analogues. Thus, it might be assumed that calcium is necessary in the later stages, from the more complex precursor state than the amino acid-stage, in the biosynthetic reaction of proteinase. In the presence of available calcium, the balance between the concentrations of the carbon source and cells was important in production, and by maintaining the balance of both factors the enzymatic activity produced was almost parallel to the cell concentrations. Furthermore, the addition of a nitrogen source promoted production about three-times.

INTRODUCTION

The previous paper[1] has shown that the production of the extracellular proteinase of *Ps. myxogenes* sp. in growing culture was remarkably affected by some components of the synthetic media: 1) Ca was an indispensable agent, 2) a rather high concentration of glucose was required for the promotion of the production, 3) supplementation of growth promoting agents, such as $MgSO_4$ or corn steep liquor, in an excess amount suppressed production. Moreover, it was pointed out that the proteinase did not appear until glucose had been used up from the media in growing culture.

In order to study whether the above factors are concerned with the net-proteinase production or not, it was desirable to use a cell suspension in production. And, it was soon shown that proteinase is produced in the absence of growth by shaking the flasks containing a cell suspension in a phosphate buffer of pH 7.0 with a carbon source and calcium ion under aerobic conditions. Further studies were conducted on various factors affecting the enzyme production. From observations on the behavior of calcium and other factors in production by cell suspensions, the phenomenon observed in growing culture was taken into account.

METHODS

The medium used to obtain cells for proteinase production had the following composition: glucose, 4%; $(NH_4)_2HPO_4$ and $Na_2HPO_4 \cdot 12H_2O$, 1%; KH_2PO_4, 0.2%; $MgSO_4 \cdot 7H_2O$, 0.05%; pH 7.0. Resting cell suspensions were obtained by growing *Ps. myxogenes* sp., inoculated from a Bouillon-agar culture, in 100 cc. of this medium in a 500-cc. shaking flask kept on a shaker at 30°C for 16 hrs. Harvesting was accomplished by centrifugation, cells washed thrice with distilled water, the precipitated material being removed by mild centrifugation and the final suspension adjusted to a concentration of 10 mg. cell material (as dry weight) per one cc. with distilled water. The yield of cells from 100 cc. of this culture was about 300 mg. (dry weight).

---

[1] K. Morihara, This Bulletin, **20**, 243 (1956).

To test the proteinase production, one cc. of this cell suspension was added into each solution containing various reagents, adjusting the final volume to 5 cc. and the mixture aerated on a reciprocal shaker of 7 cm amplitude and 130 r.p.m. at 30°C. Proteolytic activity produced in the reaction mixture was measured at various intervals by liquefaction of gelatin using the supernatant, as shown in the previous paper1). For the examination of the rate of growth, the reaction mixture was diluted to one-tenth with distilled water, and the turbidity was determined at 530 mμ using a Beckmann photometer. The diluted reaction mixture which showed a turbidity equivalent to the optical density of 0.45, contained approximately 0.2 mg. of the dried cells per one cc.

The fructose content was determined by the method of Bertrand, and the amino acid content by the Ninhydrin method2). The oxygen uptake of cells in various carbon sources was measured with the Warburg apparatus, containing 0.2 cc. of 0.05 M substrate in the side arm, 1.5 cc. of 0.06 M phosphate buffer of pH 7.0 and 5 mg. of cells as dry matter in the main compartment, 0.2 cc. of 20% KOH in the centre well of the vessel, 3 cc. in total volume being shaken at 37°C in air. Oxygen uptake was calculated by subtracting the value of autorespiration, and expressed as Q_{O_2} (μl. of oxygen uptake/hr./mg. cells).

RESULTS

I. General conditions for the enzyme production in washed cell suspensions.

1) Effects of calcium and carbon sources. The cell suspensions were prepared from the cultural medium lacking $CaCO_3$ as described above. Attempts to induce the proteinase biosynthesis were made by shaking the cell suspensions with various reagents under aerobic conditions. The results are shown in Fig. 1. In the absence of $CaCl_2$ little production was observed, while in the reaction mixture containing 10^{-3}M $CaCl_2$ without accompanying the growth, the development of activity began within four hours and gradually accerelated to reach a steady vigorous rate within 20 hours. However, even in the presence of $CaCl_2$ (10^{-3}M), the addition of calcium-removing agents such as EDTA* (10^{-3}M), Na-oxalate (5×10^{-3}M), Na-citrate (5×10^{-2}M) and NaF (5×10^{-2}M) inhibited production. It might be presumed that these agents combined with calcium, making calcium unavailable to the cells. Interestingly, as seen in Fig. 2, the inhibitory effect is seen at every stage of production, that is, the development of the production is immediately stopped by the addition of a calcium-removing agent (EDTA, 10^{-3}M) which was shown not to affect the stability of the proteinase which had already been produced. The inhibition, however, was recovered by increasing the concentration of $CaCl_2$, i.e. 5×10^{-3}M $CaCl_2$ to 10^{-3}M EDTA. On the contrary, after shaking the reaction mixture without $CaCl_2$, $CaCl_2$ was added at various intervals and its effect was studied (Fig. 3). In any case, production by the addition of $CaCl_2$ was not observed immediately but after a lag period. By shaking the mixture without $CaCl_2$ for four hours, and then adding $CaCl_2$, the maximum activity attained was the same as that when $CaCl_2$ was added initially, whilst when $CaCl_2$ was added after more than an 8-hour shaking period the maximum activity attained was lowered in spite of

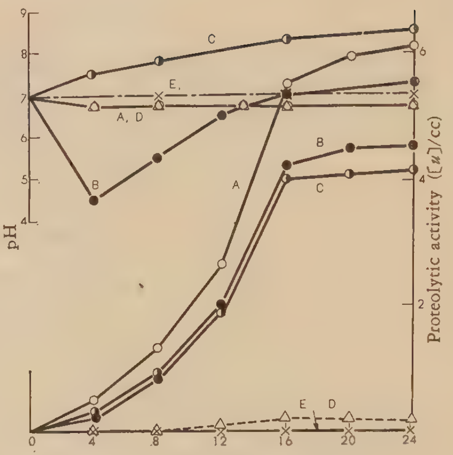


FIG. 1. Effect of Various Reagents in Proteinase Production by Cell Suspensions of *Ps. myxogenes* sp.

Five cc of the reaction mixture contained 10 mg of cells (as dry weight), M/50 phosphate buffer of pH 7.0, and the others as follows:

A, 0.2% fructose, 10^{-8}M $CaCl_2$ (○——○)
B, 0.2% glucose, 10^{-8}M $CaCl_2$, 5 mg $CaCO_3$ (●——●)
C, 0.2% Na-pyruvate, 10^{-8}M $CaCl_2$ (◑——◑)
D, 0.2% fructose (△----△)
E, 10^{-8}M $CaCl_2$ (×—·—×)

---

2) S. Moore, W. H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).

* ethylenediamine tetraacetate-Na.

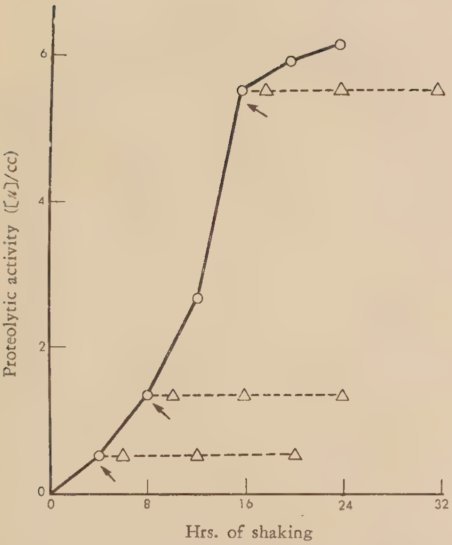


FIG. 2. Effect of EDTA in Proteinase Production.

Five cc. of the reaction mixture contained 10 mg. of cells (as dry weight), M/50 phosphate buffer of pH 7.0, 0.5% fructose and 10^{-3}M $CaCl_2$, and was shaken (○——○). At the various steps of the reaction, shown by the arrows in the figure, 0.05 cc. of 10^{-1}M EDTA was added into the reaction mixture and then the reaction was continued (△----△).

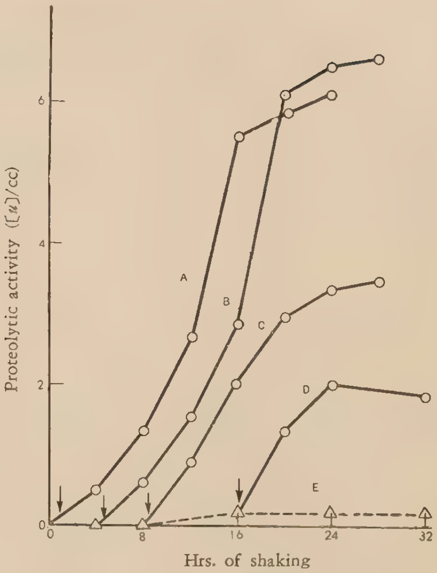


FIG. 3. Effect of Calcium in Proteinase Production.

Five cc. of the reaction mixture contained 10mg. of cells (as dry weight), M/50 phosphate buffer of pH 7.0 and 0.5% fructose, and was shaken (E, △——△). At various intervals of shaking, i.e. 0 (A), 4 (B), 8 (C) and 16 (D) hours as shown by the arrows in the figure, 0.05 cc. of 10^{-1}M $CaCl_2$ was added into the reaction mixture, and further the reaction was continued (○——○).

shortening the lag period to some extent. Perhaps the material which may be transformed into the enzyme in cells might be utilized in spite of the non-production of the enzyme. By further study, it was shown that the rate of utilization of fructose was unchanged with or without $CaCl_2$. Accordingly, it is difficult to consider that $CaCl_2$ is concerned in the metabolism of carbon source.

The indispensable effect of $CaCl_2$ was substituted with $SrCl_2$ but not by other ions such as $BaCl_2$, $MgCl_2$, $CoCl_2$, $MnCl_2$, $NiCl_2$, $ZnCl_2$ and $CdCl_2$ (Table I). From the table, it is apparent that the effect of $CaCl_2$ or $SrCl_2$ is developed by the cathion.

The production, similarly, does not occur in the absence of a carbon source, and also, the absence of phosphate. As a carbon source (activity*[1], expressed as [*u*]/cc., shown in parenthesis), fructose (5.5) could be replaced with other substances such as glucose (4.2), ethanol (3.2), gluconate (4.2), pyruvate (4.0), succinate (4.0), fumarate (4.2), lactate (4.2) etc., and various amino acids such as histidine (8.1), glutamate (5.3), aspartate (5.6), leucine (3.5), and casein hydrolysate*[2] (4.0) etc., but not by starch, dextrin, glycogen, sucrose, lactose, cellobiose, maltose, galactose, mannose, ribose, arabinose, xylose etc. and various proteins which were not metabolized by the cells. While, citrate, acetate, various sugar alcohols such as glycerol, mannitol and various peptides are slightly effective in enzyme production. The pH of the reaction mixture, however, could not be kept constant at pH 7.0 during the reaction with some useful carbon sources: By using glucose (or ethanol), the pH of the reaction mixture was lowered to a value below 5.0 within four hours' reaction, thereby inhibiting the production completely (so that the addition of $CaCO_3$ was required to keep the pH at neutrality in the reaction mixture), while with pyruvate the pH rose above 8.0, at which the production was not optimum as will be shown later. The difference in activity by different carbon sources may be reasoned partly by the change of pH during reaction. It is of interest to note that glucose which showed a high value of oxygen consumption ($Q_{O_2}=126$) was not so

---

*[1] The activity measured after shaking for 16 hrs. in the reaction mixture containing 10 mg. of cells (as dry weight), M/50 phosphate buffer of pH 7.0, 10^{-3} M $CaCl_2$ and various carbon sources at their optimal concentrations. In the reaction mixture containing glucose or ethanol, 5 mg. of $CaCO_3$ was added for neutralization.

*[2] "KAZAMINOSAN" by Nissan Co.

TABLE I. EFFECT OF VARIOUS METAL IONS ON ENZYME PRODUCTION

Five cc. of the reaction mixture contained 10 mg. of cells (as dry weight), M/50 phosphate buffer of pH 7.0, 0.2% of fructose and various metal ions as shown in the table, and shaken for 16 hrs.

Metal	(M)	Enz. activity ([u]/cc.)	Metal	(M)	Enz. activity ([u]/cc.)	Metal	(M)	Enz. activity ([u]/cc.)
None		0.2	$BaCl_2$	10^{-5}	0.3	$NiCl_2$	10^{-3}	0.0
$CaCl_2$	10^{-3}	5.5	$MgCl_2$	10^{-3}	0.5		10^{-5}	0.5
$SrCl_2$	10^{-2}	6.2		10^{-5}	0.4	$ZnCl_2$	10^{-3}	0.2
	10^{-3}	〃	$CoCl_2$	10^{-3}	0.0		10^{-5}	〃
	10^{-4}	0.7		10^{-5}	0.6	$CdCl_2$	10^{-3}	〃
	10^{-5}	0.3	$MnCl_2$	10^{-3}	0.0		10^{-5}	〃
$BaCl_2$	10^{-3}	0.5		10^{-5}	0.5			

effective as fructose which had a low value ($Qo_2=40$).

On the other hand, even when the suitable reagents mentioned above were contained production was not observed under anaerobic conditions. Further study proved the reaction to be inhibited almost completely by various metabolic inhibitors, such as KCN, NaN_3, methylene blue, monojodacetate, arsenite, dinitrophenol, α,α'-dipyridyl (10^{-3}M, respectively) and streptomycin and chloramphenicol (10 γ/cc. respectively), but not by penicillin G (100 γ/cc.). Fukumoto et al.[3] have shown that the inhibitory effect of chloramphenicol on amylase production of *B. amyloliqueficans* is diminished by addition in a later stage of reaction, but in this case such a phenomenon was not observed.

2) Effects of pH and phosphate. At various pH values, adjusted with phosphate and Tris-buffer* solutions, the production in cell suspensions was studied as shown in Fig. 4. Using these buffer solutions in the reaction mixture containing fructose, the change of pH throughout the reaction was inconsiderable except with M/50 phosphate buffer at the alkali-side. The figure shows that the optimum pH is approx. pH 7. High concentration of buffer solution revealed to suppress the production; in M/5 phosphate buffer a suppression in production was observed and also, the optimum pH shifted to 6.0.

It is of interest to note that a difference in production is observed with or without phosphate, especially at pH 6.0. Fukumoto et al.[4] have found that amylase production by cell suspensions of *B. amyloliquefícans* could hardly be observed without phosphate when sugars were used as the carbon source, while a slight production was seen in the case of organic acid salts. In order to clarify this problem, the effects of various concentrations of phosphate in proteinase production were studied at various pH values, adjusted with M/50 Tris-buffer (Table II).

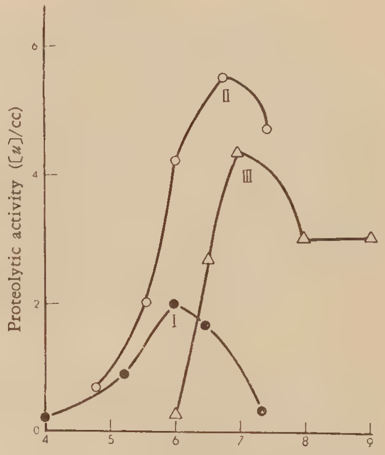


FIG. 4. Effect of pH in Proteinase Production.

Five cc. of the reaction mixture contained 10mg. of cells (as dry weight), 0.2% fructose, 10^{-3}M $CaCl_2$ and various buffer solutions varying the pH values;
I. M/5 phosphate buffer (●——●)
II. M/50 phosphate buffer (○——○)
III. M/50 Tris-buffer (adjusted by HCl, △——△)
The reaction was done for 16 hrs.

From the table, it can be seen that the effect of phosphate is observed remarkably at pH 6, slightly at 7 and not at 8, with the concentration of the phosphate at over 10^{-3}M. And also, the utilization of fructose is shown to be almost parallel with production in various pH values with or without phosphate.

It is a well-known fact that phosphorus plays an important role in metabolism, that is, the oxidative phosphorylation in living organisms. Then, the ques-

---

[3] J. Fukumoto, T. Yamamoto, D. Tsuru, *J. Agr. Chem. Soc. Japan*, **31**, 545 (1957).

* Tris (hydroxymethyl) amino methane.

[4] J. Fukumoto et al., *J. Agr. Chem. Soc. Japan*, **31**, 425 (1957).

TABLE II. EFFECT OF PHOSPHATE AT VARIOUS pH RANGES

Five cc. of the reaction mixture containing 10 mg. of cells (as dry weight), 0.5% fructose (25 mg.), 10^{-3} M $CaCl_2$, M/50 Tris-buffer adjusted at pH 6, 7 and 8, and various concentrations of phosphate adjusted in each pH value as shown in the table was shaken for 16 hrs.

Phosphate concn. (M)	10^{-2}	10^{-3}		10^{-4}	10^{-5}	0	
pH	Enz. activity ([u]/cc.)	Enz. activity ([u]/cc.)	Fructose utilized (mg.)	Enz. activity ([u]/cc.)	Enz. activity ([u]/cc.)	Enz. activity ([u]/cc.)	Fructose utilized (mg.)
6	4	4	11.2	3.5	0.5	0.5	2.6
7	5.5	5.5	12.7	4.6	4.5	4.5	10.1
8	3	3	11.7	3	3	3	11.2

tion may arise as to whether coupled phosphorylation does not occur in production at pH 8. To clarify this question the effect of dinitrophenol (DNP), which is known as an inhibitor of oxidative phosphorylation, was studied at various pH values. The results showed that the production was inhibited almost completely by the addition of 10^{-3}M DNP in the reaction mixture containing 10^{-3}M phosphate at all pH ranges.

These results suggest that production could occur only when coupled with the oxidative phosphorylation at all pH ranges; in other words, phosphorus is necessitated even at pH 8. It has already been pointed out⁵⁾ that organic phosphorus kept within the cell might be used for the production of amylase in cell suspensions of mould. By adopting this view, it might be said that the difference in the requirement of phosphate is exclusively attributed to the difficulty or ease in the utilization of phosphorus kept in the cell-body by varying the pH-values.

3) Effect of nature of cells. It was determined whether the enzyme producing ability of the cells is affected by differences in nutrition or age. Even by varying the concentration of glucose, with or without $CaCO_3$, or adding an excess amount of growth promoting agent such as $MgSO_4$ or corn steep liquor in

TABLE III. ENZYME PRODUCING ABILITIES OF CELLS CULTIVATED IN DIFFERENTIATED MEDIUM

The basic medium contained 1% $(NH_4)_2HPO_4$ and $Na_2HPO_4\cdot 12H_2O$, 0.2% KH_2PO_4, and 0.05% $MgSO_4\cdot 7H_2O$, with other factors as shown in the table. After shaking culture (100 cc of these media in 500 cc. shaking flasks) for 16 hrs. at 30°C, the cell suspensions were prepared as described in the paragraph: "Method".

The production was assayed as usual, using cell suspensions of 10 mg. (as dried cell) in M/50 phosphate buffer of pH 7.0 containing 0.2% fructose and 10^{-3}M $CaCl_2$, and shaken for 16 hrs. Cells yielded was shown as dry weight.

"MEDIUM" for obtaining cells					
Glucose %	$CaCO_3$ %	C.S.L. %	$MgSO_4$ %	Cells yielded (mg/100 cc.)	Enz. activity ([u]/cc.)
1	0.05			280	4.4
〃				200	5.4
2	0.2			250	5.5
4	0.5	0.05		380	〃
〃				300	〃
7	2.0	0.2		654	5.2
〃			0.45	810	6.0

TABLE IV. EFFECT OF AGE ON CELLS

The medium used was 4% glucose, 1% $(NH_4)_2HPO_4$ and $Na_2HPO_4\cdot 12H_2O$, 0.2% KH_2PO_4, 0.05% $MgSO_4\cdot 7H_2O$ with or without 0.5% $CaCO_3$. The shaking culture and production in cell suspensions was performed by the same method as shown in Table III. Cells yielded was shown as dry weight.

Cells obtained from the medium with $CaCO_3$

Age (hrs.)	pH	Cells yielded (mg/100 cc.)	Enz. activity ([u]/cc.)
20	6.0	334	5.5
44	6.5	374	〃
68	7.0	478	〃
92	〃	425	0.7
100	〃	353	0.5

Cells obtained from the medium without $CaCO_3$

Age (hrs.)	pH	Cells yielded (mg/100 cc.)	Enz. activity ([u]/cc.)
16	6.0	300	5.9
20	5.5	310	5.5
44	4.0	555	1.3
96	3.0	715	0.5

---

5) O. Tanabe and K. Tonomura, *Bull. Ferm. Res. Inst. Japan*, **9**, 29 (1953).

the synthetic medium, in which proteinase production was profoundly affected in growing culture as shown in the previous paper[1)], the proteinase producing abilities of cells prepared from these media were shown to undergo only a slight change (Table III). However cells harvested from natural medium such as bouillon or peptone possessed little producing ability even when not only fructose, but also pyruvate was used as a carbon source.

The effect of age is shown in Table IV. Cells obtained from a medium containing 4% glucose without $CaCO_3$, cultured for more than 44 hrs. showed a remarkable decrease in enzyme producing ability owing to the acidification of the medium, while, in the presence of $CaCO_3$ in the medium producing ability was almost unchanged for 68 hrs. and then decreased remarkably along with the lysis of cells.

II. Mutual relation between the enzymatic activity produced and the concentration of each component necessitated in production by cell suspensions.

1) Effects of concentrations of carbon sources and calcium ion. Fig. 5 shows the effects of concentrations of various carbon sources in proteinase production of resting cells. From the figure, it may be seen that a stronger inhibition occurs along with the increase

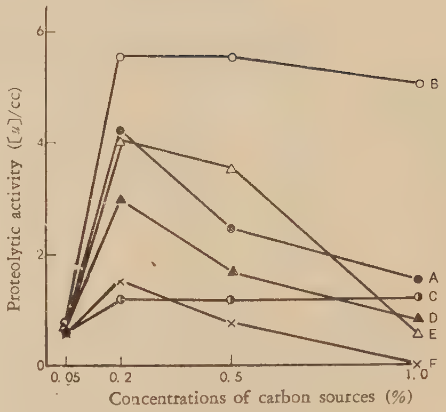


FIG. 5. Effects of Concentrations of Various Carbon Sources.

Five cc. of the reaction mixture contained 10 mg. of cells (as dry weight), M/50 phosphate buffer of pH 70, 10^{-3}M $CaCl_2$ and in varying the concentrations of the various carbon sources:

A, glucose (5–10 mg. $BaCO_3$ was added in accompanying with the increase of the concentrations from 0.2% to 1.0%, (●——●)
B, fructose (○——○)
C, glycerol (◑——◑)
D, ethanol ($BaCO_3$ was added as samely as in A, ▲——▲)
E, Na-gluconate, Na-lactate, Na-pyruvate, Na-fumarate or Na-succinate (△——△)
F, Na-citrate (×——×)
The reaction was done for 16 hrs.

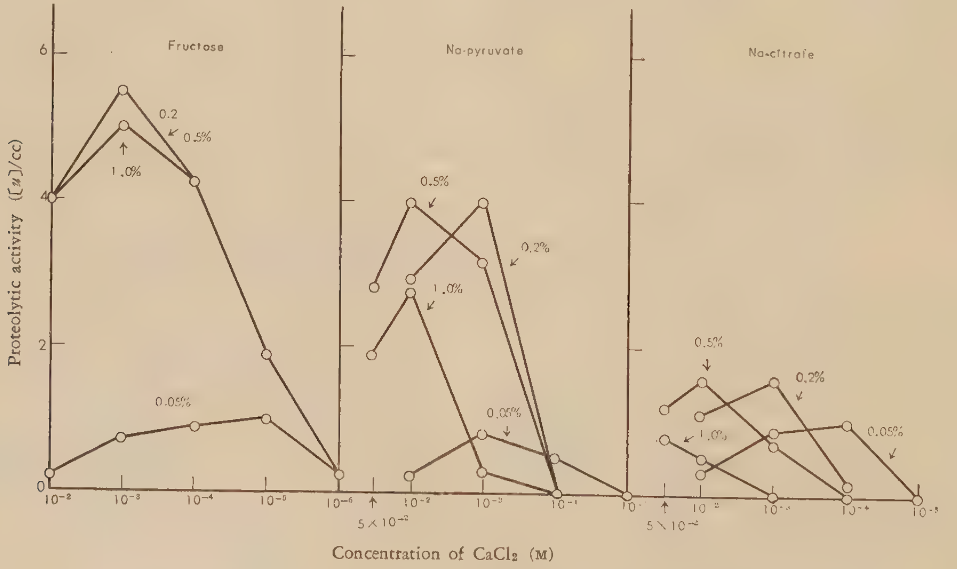


FIG. 6. Effect of Concentrations of $CaCl_2$ in Various Amounts of Carbon Sources.

The reaction mixture contained 10mg of cells (as dry weight), M/50 phosphate buffer of pH 7.0, and the others as shown in the figure, and was shaken for 16 hrs.

of the concentration of various carbon sources, such as glucose, ethanol, gluconate, pyruvate, lactate, succinate, fumarate and citrate, but it is not so strong in the case of fructose and glycerol. This result might lead to the consideration that inhibition occurred because of the lack of utilizable calcium by combination with organic acids, i.e., 1) in glucose or ethanol, where a large amount of acids is formed (thus making the addition of $CaCO_3$ or $BaCO_3$ necessary in the reaction mixture, for neutralization) the inhibition is more remarkable in comparison with that of fructose or glycerol, where much less acids are formed. 2) All of the various organic acids inhibit production at high concentrations. Thus, the following experiment on the effect of the concentration of $CaCl_2$ in varying concentrations of organic acids and fructose was carried out for comparison. The results are shown in Fig. 6. As expected, the inhibition occurring in one percent of citrate or pyruvate is shown to be recovered by increasing the concentration of calcium, that is, in the former 5×10^{-2}M, and in the latter 10^{-2}M is necessitated as optimal concentration. Namely, it might be said that some carbon sources, especially various organic acids, are not only utilized as carbon sources in production at low concentrations, but also as calcium-removing agents at their high concentrations. Therefore, the optimum concentration of calcium is differentiated by the kinds of carbon sources and their concentrations.

TABLE V. EFFECT OF CELL CONCENTRATIONS IN PROTEINASE PRODUCTION

The reaction mixture consisted of M/50 phosphate buffer of pH 7.0, 10^{-3}M $CaCl_2$ (in 40 mg. of cells, 10^{-2}M $CaCl_2$ is used), and various concentrations of cells and fructose as shown in the table, making a total volume of 5 cc., and was shaken for 16 hrs.

Fructose concentration % (mg.)		Cell concentration (mg. as dry weight)				
		5	10	20	30	40
0.05 (2.5)	Enz. activity ([*u*]/cc.)	1.0	0.5	0		
	Fructose utilized (mg.)	2.5	2.5	2.5		
0.2 (10)	Enz. activity ([*u*]/cc.)	2.7	5.5	3.0	0.5	0
	Fructose utilized (mg.)	5.2	10	10	10	10
0.5 (25)	Enz. activity ([*u*]/cc.)	2.5	5.5	10.5	6.8	4.5
	Fructose utilized (mg.)	5.0	12.7	25	25	25
1.0 (50)	Enz. activity ([*u*]/cc.)	2.0	5.0	11.0	17.0	23
	Fructose utilized (mg.)	4.6	12.3	25	38.3	50

2) Effect of concentrations of cells. Although it has been shown that a rather high concentration of glucose is required for the promotion of enzyme production in growing culture, such a phenomenon was not recognized in production experiments by cell suspensions. Moreover, we have not found such specific promoting substance among various carbon sources that could be designated as an "inducer" for enzyme production in cell suspensions. Thus, the effects of cell concentrations in production were studied, as seen in Table V, when the concentration of cells was in a range of 5 to 40 mg., activity produced was almost parallel to the cell concentrations in the reaction mixture containing 1.0% fructose; this was also observed in the range of 5 to 20 mg. in a mixture containing

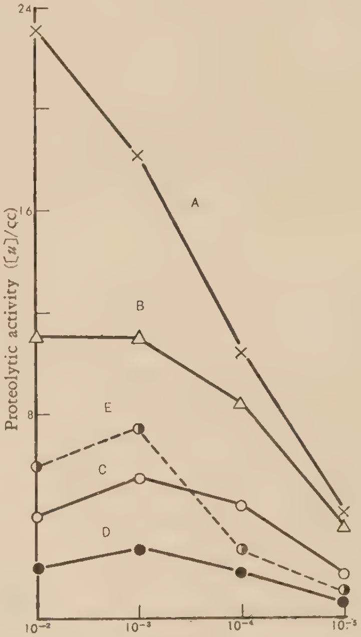


FIG. 7. Effect of Concentrations of $CaCl_2$ in the Reaction Mixture Containing Various Contents of Cells.

The reaction mixture contained M/50 phosphate buffer of pH 7.0, various concentrations of $CaCl_2$ as shown in the figure, and the others as follows;

A, 40 mg. of cells (as dry weight), 1.0% fructose (×——×)
B, 20 mg. of cells (as dry weight), 0.5% fructose (△——△)
C, 10 mg. of cells (as dry weight), 0.5% fructose (○——○)
D, 5 mg. of cells (as dry weight), 0.5% fructose (●——●)
E, D+0.1% $(NH_4)_2HPO_4$ (◐——◐)
The reaction was done for 16 hrs.

0.5% fructose and in the range of 5 to 10 mg. in a mixture containing 0.2% fructose. Further, the utilization of fructose is also parallel to the cell concentrations. However, the higher the cell concentrations in comparison to the concentration of fructose, i.e., when the cell concentration is 30 mg. or above in 0.5% fructose and above 20 mg. in 0.2% fructose, the higher inhibition is observed.

On the other hand, the required concentration of calcium was not changed by increasing the cell concentration in a range of 5 to 20 mg. (in five cc. of the reaction mixture) not-withstanding the addition of a nitrogen source, but when the cell concentration was 40 mg. a higher concentration (10^{-2}M) was required to obtain a maximum activity (Fig. 7).

From the above results, it may be concluded that the enzymatic activity produced in cell suspensions, is controlled by the ratio of the concentration of the carbon source and the cells, when available calcium is present. By keeping both of them in balance, activity produced is proportional to the cell concentrations in the reaction mixture.

III. Effects of nitrogen sources and amino acid analogues.

The following experiment was conducted on the effects of various nitrogen sources in production, by cell suspensions containing other reagents which were essential for production as mentioned above (Fig. 8). It may be seen that the addition of a nitrogen source such as $(NH_4)_2HPO_4$ or leucine promotes the production to about three times of that without the supplement of a nitrogen source, with a slight increase of growth and utilization of fructose (65 and 75% utiliza-

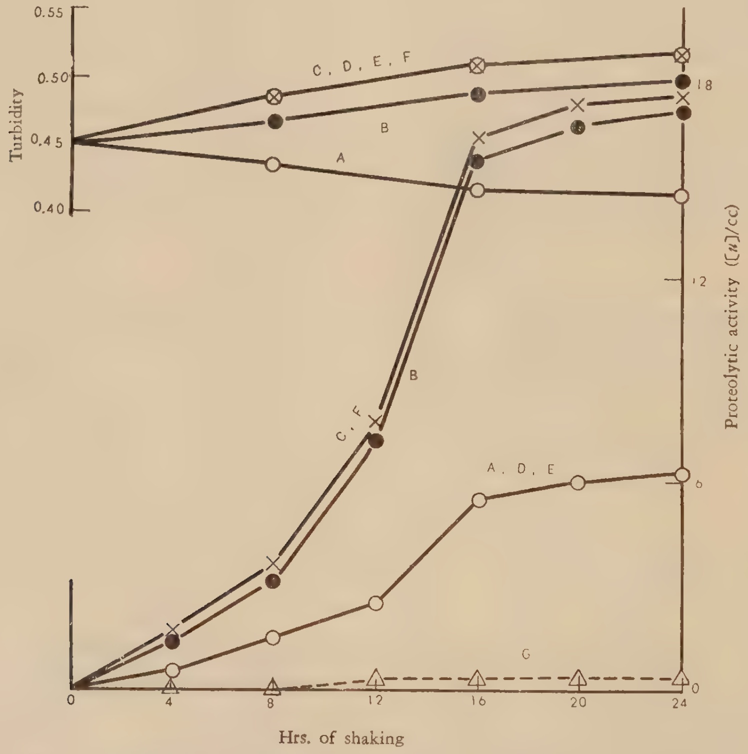


FIG. 8. Effects of Nitrogen Sources and Amino Acid Analogues on Proteinase Production.

Five cc. of the reaction mixture contained 10 mg. of cells (as dry weight), M/50 phosphate buffer of pH 7.0, 0.5% fructose, 10^{-3}M $CaCl_2$ and various nitrogen sources as follows:

A, no addition (○——○)
B, 0.1% $(NH_4)_2HPO_4$ (●——●)
C, 0.1% L-leucine (×——×)
D, 0.1% DL-norleucine (○——○)
E, 0.1% $(NH_4)_2HPO_4$, 0.1% DL-norleucine (○——○)
F, 0.1% L-leucine, 0.1% DL-norleucine (×——×)
G, omitted 10^{-3}M $CaCl_2$ in each flask, respectively (△----△)

tion, in the experiment shown in the figure in the presence of $(NH_4)_2HPO_4$ and leucine respectively in comparison with 50% utilization without a nitrogen source). This promoting effect was also observed with other nitrogen sources, such as NH_4Cl, $NaNO_3$ as inorganic nitrogen sources, various amino acids and yeast extract etc. Nevertheless $(NH_4)_2SO_4$, $NaNO_2$, cysteine, cystine and methionine had rather inhibitive effects on production and various protein nitrogen sources did not affect production.

Norleucine, however, when added to the reaction mixture containing $(NH_4)_2HPO_4$, was shown to diminish activity, to a point corresponding to the activity in the absence of $(NH_4)_2HPO_4$, that is, it retracted the promoting effect of the nitrogen source, while it did not diminish the promoting effect of leucine, which is homologue. However, norleucine itself does not affect the production at all in the reaction mixture not containing other nitrogen sources. It was ascertained by further study, that norleucine is utilized by the cells in spite of the presence or absence of other nitrogen sources, by determination of the amino acid content by the Ninhydrin method and by paper-partition chromatography.

The same phenomenon as seen in the case of norleucine, was also observed with other amino acid analogues such as norvaline, 5-methyl-tryptophane and β-hydroxy glutamic acid. The latter two, however, diminished the promoting effect to a certain extent in the case their homologue amino acids are used as the nitrogen source.

IV. Others.

The following study was attempted to investigate the effects of various anions and cathions in production. It was found that production is inhibited by the addition of Cu, Co, Fe, Sn and Ni but not affected by Zn, Mg, Cd, Ba, Mn and Sr at concentrations of 10^{-3}M, respectively. Among the substances used as anions, remarkable inhibition was observed with those containing sulfur in concentrations of 10^{-4}M, as shown in Table VI. Further study, revealed that the SO_4 ion does not only affect the activity of the proteinase but is also an indispensable agent for the growth of bacteria. It is however obscure, how this inhibition occurred. Accordingly, the inhibition by $(NH_4)_2SO_4$, cystine, cysteine or methionine mentioned above might be due to the sulfur component. Anions such as Cl, Br, I, F, BO_3, CO_3 in concentrations of 10^{-3}M did not affect production.

TABLE VI. EFFECTS OF VARIOUS SUBSTANCES CONTAINING SULFUR ON PROTEINASE PRODUCTION

Five cc. of the reaction mixture contained 10 mg. of cells (as dry weight), M/50 phosphate buffer of pH 7.0, 0.2% fructose, 10^{-3}M $CaCl_2$ and various substances shown in the table, and it was shaken for 16 hrs. as usual.

Substances	(M)	Inhibition (Percentage)
Na_2SO_4	10^{-3}	82
	10^{-4}	〃
	10^{-5}	0
$NaHSO_3$	10^{-3}	82
	10^{-4}	〃
$Na_2S_2O_3$	〃	84
$(NH_4)_2SO_4$	〃	71
$MgSO_4$	〃	82

Further experiments were carried out to investigate the effect of base analogues. 8-Azaguanine (5×10^{-3}M) did not inhibit the production but rather accerelated it, similarly to the addition of a nitrogen source, while it suppressed growth completely in growing culture. However, the presence of a nitrogen source seemed to diminish the effect partly as seen in Table VII. Though thiouracil (5×10^{-3}M) was shown to inhibit production remarkably, the reason for inhibition i.e., whether it is caused by the sulfur component or not is obscure.

DISCUSSION

The above data shows that the proteinase

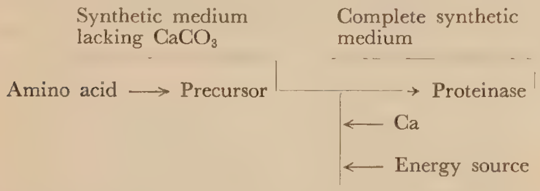
TABLE VII. EFFECT OF BASE ANALOGUES ON PROTEINASE PRODUCTION

The conditions are the same as in Table VI.

Base analogues	(M)	Others	Final pH	Enz. activity ([u]/cc.)
No addition			6.8	5.5
8-Azaguanine	5×10^{-3}		7.1	13.0
〃		$(NH_4)_2HPO_4$ 0.1%	〃	9.5
Thiouracil	5×10^{-3}		6.3	1.0
		Uracil 5×10^{-3}M	6.7	11.0
Thiouracil	5×10^{-3}	〃	6.4	6.0

production in cell suspensions of *Ps. myxogenes* sp. occurs in the presence of a carbon source, phosphate (differentiated by pH values), and calcium ion under aerobic conditions.

However, this reaction is not affected by amino acid analogues, which are generally known[6,7,8] to inhibit the incorporation of corresponding amino acids into the cellular protein. Therefore, it may be assumed that the production of resting cells in the absence of nitrogen source does not involve free amino acid utilization, but rather utilizes the preformed complex precursor substance, which is formed within the cells grown in the synthetic medium without the addition of calcium. Thus, calcium might be necessary in any step of the biosynthetic reaction from the precursor state, together with metabolism of a carbon source which perhaps might be the energy supplier in proteinase production. The following scheme may be advanced to explain:



On the role of calcium in production, it is difficult to assume that calcium is involved in the metabolism of the cells, from experimental results showing that no difference is observed in the utilized ratio of carbon source either in its presence or absence.

From observations on the behavior of calcium and other factors in production by cell suspensions, it might be possible to explain the various phenomena seen in the proteinase production by growing culture. When glucose is used as carbon source, it is metabolized very rapidly so that its intermediary products (various organic acids) are fastly formed before they can be used up. Accordingly, it might be considered that intermediary products of glucose may combine with calcium, as observed in the experiments using various organic acids such as gluconate, pyruvate, succinate, fumarate, citrate etc., thus making calcium unavailable to the bacteria, as has been suggested by Mandels and Reese[9] on the cellulase production by mould. Upon consumption of these intermediary products after the disappearrance of glucose, calcium would be released, this being essential for proteinase production. This might be an answer for the question why proteinase does not appear until glucose is used up in the medium in growing culture.

Accordingly, the production in growing culture would be affected by the conditions, at the time when calcium is released, and not by the natures of cells as seen in this paper. This corresponds to the time when normal cellular multiplication no longer takes place as shown by Nomura et al.[7] in amylase formation of *B. subtilis*. Thus, the production in growing culture may be regarded to be similar to that of cell suspensions described above.

The enzymatic activity produced in cell suspensions is controlled by the ratio of the concentrations of the carbon source and the cells. When the balance of both factors is kept in suitably, the enzymatic activity produced is almost parallel to the cell concentrations, while in the unbalanced condition, a lower production of enzyme is obtained. Therefore, the remarkable increase in production along with the increase in the concentrations of glucose suitably supplemented with other factors in growing culture might be occurred by the increase in concentrations of cells simultaneously maintaining the balance optimally with the concentrations of carbon sources, which must be intermediary products of glucose. On the other hand, a remarkable decrease in production by the addition of a growth promoting agent, even at a high con-

---

[6] H. O. Halvorson and S. Spiegelman, *J. Bact.*, **64**, 207 (1952), **65**, 496, 601 (1953).

[7] M. Nomura et al., *J. Biochem.* **43**, 841 (1956).

[8] J. Fukumoto et al., *J. Agr. Chem. Soc. Japan*, **31**, 545 (1957).

[9] M. Mandels and E. T. Reese, *J. Bact.*, **73**, 269 (1957).

centration of glucose in the growing culture, might be caused by an unbalance of both factors, i. e., the lack of carbon sources for production.

This assumption leads to the next consideration that factors affecting the growth and proteinase production are not always the same. The following facts give support to a difference between these factors: 1) Calcium is essential for production but is not necessarily essential for growth, 2) SO_4 ion inhibits production remarkably, while it is required as an indispensable agent for growth, 3) 8-Azaguanine does not only inhibit but rather accerelates production, while growth is inhibited completely.

SUMMARY

Factors affecting the proteinase production in cell suspensions of *Ps. myxogenes* sp. were studied and the following facts are shown:

1) The production was observed in cell suspensions in a phosphate buffer of pH 7.0, containing a carbon source and calcium ion under aerobic conditions, without accompanying growth. The calcium ion could be replaced with strontium but not by others, and inhibition was observed in the presence of calcium-removing agents such as EDTA, Oxalate, Citrate and NaF. The carbon sources which were to be utilized by the cells were all found to be effective, and production was inhibited by various metabolic inhibitors.

2) The optimum pH was about 7.0, and the promoting effect of phosphate was observed remarkably at pH 6 and slightly at 7 but not at 8.

3) The enzyme producing abilities by nutritionally differentiated types of cells prepared from synthetic media (of which the components were differentiated) in various concentrations of glucose (1–7%), in the presence or absence of $CaCO_3$ or excess addition of a growth promoting agent, were almost identical with one other. Furthermore ability was not altered throughout the stationary phase when the pH of the cultural medium was kept at neutrality.

4) The production was more inhibited along with the increasing of the concentrations of carbon sources such as organic acids and glucose or ethanol in which were observed high acid formation, but not in fructose or glycerol in which low acid formation was seen. The inhibition, however, was recovered to some extent by the further addition of calcium. In other words, it could be said that the optimum concentration of calcium was differentiated by the sorts of carbon sources or by their concentrations.

5) The balance between the concentrations of carbon source and cells was important for the production, and when kept the balance of both factors suitably the enzymatic activity produced was parallel to the concentrations of cells.

6) The addition of a nitrogen source in the reaction mixture promoted production about three times. However, in the presence of amino acid analogues, which did not affect the production in the reaction mixture without a nitrogen source, the promoting effect of the nitrogen source was diminished except when their homologues were used.

7) The production was inhibited remarkably by the addition of various substances containing sulfur, while 8-Azaguanine, base analogue, did not affect the production.

The author wishes to express his sincere thanks to Prof. H. Katagiri of Kyoto Univ. and also to Mr. E. Masuo of this Laboratory, for their constant guidance and encouragement in this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 1, p. 60~63, 1959]

Studies on the Protease of *Pseudomonas*

Part V. On the Role of Calcium in the Enzyme Production

By Kazuyuki Morihara

Research Laboratory of Shionogi & Co., Ltd. Amagasaki, Hyogo

Received July 17, 1958

It was ascertained by the tracer method that calcium, which has been added as an indispensable agent for proteinase production of *Ps. myxogenes* sp., is constituted in the produced enzyme itself. Also, the removal of calcium from the enzyme molecule, by treatment with M/50 EDTA at 50°C, caused inactivation. Accordingly, it can be said that the development of the enzymatic action is not appeared in the absence of calcium in the enzyme molecule, so this might be the reason why the supplementation of calcium is indispensable in proteinase production.

INTRODUCTION

Since 1928, Merrill & Clark⁽¹⁾ have found that calcium was necessitated as an indispensable agent for the production of extracellular proteinase by *B. proteus vulgaris* in synthetic medium, the same phenomenon being ascertained in various strains of bacteria by many other workers⁽²⁻⁷⁾. Moreover, the activities of these bacterial proteinases when they were separated from bacterial cells, was shown not only to be protected⁽⁵,⁶⁾ by the addition of calcium against inactivation by such treatments as dialysis, acid or heat, but also to be inactivated by the addition of various calcium-removing agents⁽⁴,⁶⁾ such as fluoride, citrate, oxalate and ethylenediaminetetraacetate (EDTA). Gorini⁽⁶⁾ has pointed out that calcium plays a fundamental role in bacterial proteinases not only in their stability but also in their activity; it is a constituent of these enzymes which must therefore be considered as metallo-proteins. And further, he claimed that calcium as a firmly bound prosthetic group is required to stabilize the proteinase, which, in the absence of calcium, was inactivated as rapidly as it was produced, contrary to the view proposed by Merrill and Clark⁽¹⁾, Haines⁽³⁾ and Beumer⁽⁴⁾ that calcium participated in the synthesis of proteinase.

Though Gorini's work was performed using a cultural filtrate of bacteria as the enzyme source, the same phenomenon such as inhibition by EDTA, oxalate etc. has been observed in crystalline proteinase of *B. subtilis* by Fukumoto et al.⁽⁸⁾.

The present author has already reported that calcium is essential in proteinase production of *Ps. myxogenes* sp. not only in growing culture⁽⁷⁾ but also in cell suspensions⁽⁹⁾. Also⁽⁹⁾, from the experiment analyzing the mechanism of enzyme production, it has been suggested that calcium might be required in any step of the synthesizing reaction from the precursor state (a more complex substance than amino acids) to enzyme, but not concerned in the metabolism of carbon source.

In the present paper, examinations were made as to whether labelled calcium added in

---

1) A. T. Merrill & W. M. Clark, *J. Bact.*, **15**, 267 (1928).
2) E. D. Wilson, *ibid*, **20**, 41 (1930).
3) R. B. Haines, *Biochem. J.* **25**, 851 (1931); **26**, 323 (1932); **27**, 466 (1933).
4) J. Beumer, *Acta biol. belg.*, **2**, 273 (1941).
5) L. Gorini and C. Fromageot, *Compt. rend*, **229**, 559 (1949).
6) L. Gorini, *Biochim. et Biophys. Acta*, **6**, 237 (1951).
7) K. Morihara, This Bulletin, **20**, 243 (1956).
8) J. Fukumoto, T. Yamamoto and K. Ichikawa, *J. Agr. Chem. Soc. Japan*, **31**, 331 (1957).
9) K. Morihara, This Bulletin. **23**, 49 (1959).

the reaction mixture containing cell suspensions of *Ps. myxogenes* sp., is constituted in the produced proteinase itself.

METHODS AND RESULTS

The washed cell suspensions of *Ps. myxogenes* sp. were prepared, as it has been shown in the previous paper[9]. Two hundred mg. of the washed cells (dry wt.) was added to the reaction mixture containing M/50 phosphate buffer of pH 7.0, 1% fructose, 0.15% $(NH_4)_2HPO_4$ and 10^{-3}M $CaCl_2$, composed of 20μC $Ca^{45}Cl_2$, to make the total mixture up to 100 cc., in 500-cc. shaking flasks, and incubated on a shaker at 30°C. for 2 days. After removing the cells from the reaction mixture by centrifugation, the crystalline proteinase was isolated from the supernatant (showing activity, about 20[u]/cc.[7]) by the following procedure as shown in the previous paper[10]; rivanol precipitation (0.1%) and elution by M/2 $CaCl_2$, fractional precipitation by acetone (40–60%), solubilization in M/100 $CaCl_2$, and inoculation of seed-crystals. Even without such treatment as salting out and dialysis, crystallization occurred very easily in this case.

The twice recrystallized proteinase was then subjected to paper-electrophoresis under the conditions seen in Fig. 1. After 5 hours' run, the paper was dried in the atmosphere. The detection of protein by "Amido-schwarz" was performed directly on guide strips (1cm wide) cut from both ends of paper, and radioautography (i.e., contact printing to X-ray film for 20 days) was made on a strip (3 cm wide) cut from half of the remaining strip. The remaining strip of paper (3 cm wide) was cut into pieces, each 0.5 cm. in length, eluted with 10 cc. of 0.5% $NaHCO_3$, and proteinase activity determined on this eluate. From the figure, it is clearly seen that three bands, i.e., colour reaction of protein, expression of X-ray film and the enzymatic activity, correspond accurately with each other. When unlabelled crystalline proteinase and $Ca^{45}Cl_2$ were mixed and subjected to paper-electrophoresis under the same conditions as in Fig. 1, the former migrated to the cathode and the latter to the anode separately. Therefore, the result in the figure shows that Ca^{45} is constituted in the proteinase molecule, and the band that migrated to the anode seen in radioautography might perhaps occur by free Ca^{45}, in which band the colour reaction even by Brom phenol blue or Ninhydrin was not observed.

Further study was conducted on the effects of calcium-removing agents on the stability of the crystalline proteinase. As it has already been shown in the previous paper[10], the enzyme activity was not altered by treatment with M/50 EDTA or M/10 oxalate in the enzyme concentration by 100 [u]/cc. in pH 7.0 at 40°C for 60 minutes. Thus EDTA was treated at 50°C. As it is seen in Fig. 2, inactivation occurs by the addition

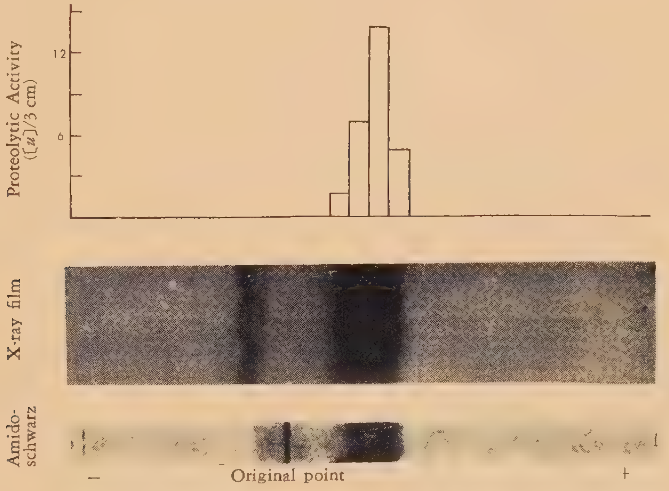


FIG. 1. Paper-electrophoresis of the Labelled Crystalline Proteinase.

The width of paper was 8 cm (length, 15 cm) and M/20 Tris-buffer (pH 8.5) containing M/100 calcium-acetate was used as buffer solution. The phoresis was done by 0.5 mA/cm (450 V) at about 5°C for 5 hours.

---

[10] K. Morihara, This Bulletin, **21**, 11 (1957)

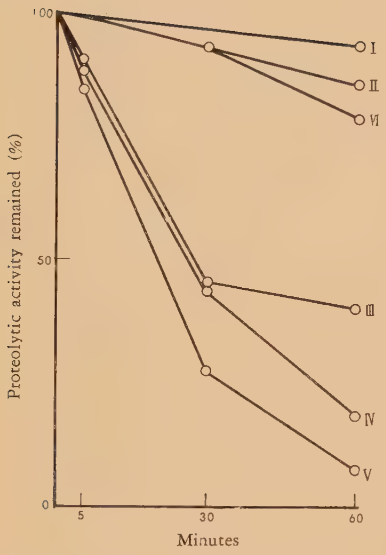


FIG. 2. Effects of some Calcium-Removing Agents on the Stability of the Crystalline Proteinase.

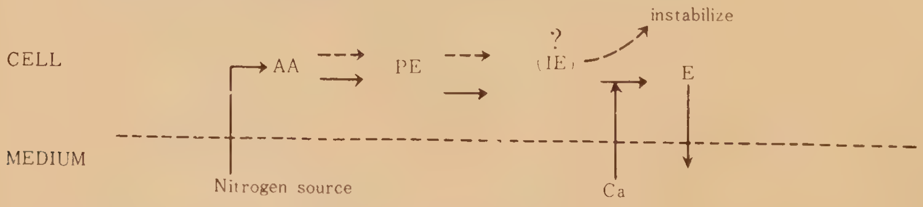
Five cc. of the reaction mixture contained the crystalline proteinase (20 [*u*]/cc.), 0.25 M Tris-buffer of pH 8.0 and some calcium-removing agents in varying concentrations as follows; I, None; II, M/10 ammonium-oxalate or -citrate; III, M/50 EDTA; IV, M/150 EDTA; V, M/500 EDTA; VI, III+M/25 $CaCl_2$. After the treatment at 50°C at various intervals, the reaction mixture was cooled immediately and the remaining activity was determined.

of EDTA even at a concentration of M/500, while this is not observed in case of M/10 NH_4-citrate. In the presence of $CaCl_2$ contained in a more excess amount than EDTA, the inactivation is protected. However, even when $CaCl_2$ was added in excess amounts (M/50) to the inactivated enzyme solution treated previously by EDTA (M/150) at 50°C for 30 minutes, the inactivated enzyme solution was not further reactivated.

DISCUSSION

Employing the tracer method, we have shown that proteinase of *Ps. myxogenes* sp. is constituted with calcium which has been added as an indispensable agent for the production. By treatment of EDTA at 50°C the enzyme is inactivated and, as it has been shown in the previous paper[10], inactivation by acid (pH 4) or heat (58°C) can be partly prevented by the addition of calcium. These phenomena may suggest that calcium as a constituent of the enzyme molecule plays an important role not only in its activity but also in its stability. Accordingly, even if the enzyme were produced in the absence of calcium, the enzyme might not develop its activity or be instabilized immediately. This might be the reason why the requirement of calcium is essential in production.

Then, the next question may arise as to why the production of the enzyme in cell suspensions is inhibited even in the presence of a weak calcium-removing agent (NaF) at 30°C as shown in the previous paper[9], while the enzyme excreted is not inactivated by treatment of EDTA at 40°C, nor by oxalate or citrate even at 50°C. It might be possible to consider that the inhibition seen in the enzyme production does not occur by inactivating the enzyme produced, but making calcium unavailable to the bacteria; suggesting the probability of intracellular synthesis of the enzyme. This leads to the further consideration that the enzyme may be excreted into the external medium immediately after it is synthesized at the intracellular site, as the enzyme has not been found to be intracellular. A tentative scheme to illustrate the proteinase synthesis in *Ps. myxogenes* sp. is shown as follows:



AA=Amino acid, PE=Precursor of enzyme, IE=Inactive enzyme (absence of calcium in the enzyme molecule), E=Active enzyme.

The fact* that the enzyme is not inactivated by treatment of M/10 NH_4-oxalate at 40°C, by which Fukumoto et al.[8)] were able to inactivate the enzymatic action of the crystalline or purified proteinase obtained from several strains of *B. subtilis* almost completely within 30 minutes, might suggest that the bond between calcium and protein in the enzyme molecule is less labile. Gorini[6)] has shown that the degrees of the inactivation of various bacterial proteinases by calcium-removing agents are different according to their origin, and Fukumoto et al.[8)] have also recently reported similar result-views.

---

* The author made a mistake in assumption that calcium is not a constituent of the enzyme, as shown in the previous paper[10)].

SUMMARY

It was ascertained by the tracer method that calcium is a constituent of the proteinase of *Ps. myxogenes* sp. However, the activity is not altered by the treatment of EDTA until the temperature reaches to 50°C. This might suggest that calcium is firmly combined to the enzyme protein. From these results, the indispensable role of calcium in proteinase production has been discussed.

The author wishes to express his sincere thanks to Prof. H. Katagiri of Kyoto Univ. and also to Mr. E. Masuo of this Laboratory, for their constant guidance and encouragement in the course of this work.

Oryzanone, an Attractant of the Rice Stem Borer

Sir:

It has been reported that after the 2, 4-D (2, 4-dichlorophenoxyacetic acid) treatment of crops there arises an increase in injurious insects1). This is also verified in case of the rice stem borer (*Chilo suppressalis* Walker) in Japan2,3).

To solve the above problem the authors propose to investigate the possiblity of developing a repellent for the rice stem borer by the application of a repellent mixed with 2, 4-D.

However, in order to find a repellent it was first necessary to find a good attractant which could then be used as a standard. If a suitable good attractant is to be found and standardized, consequently no repellent can be based against the standardized attractant.

This report concerns a study of a new naturally occurring rice stem borer attractant which the authors isolated and named Oryzanone.

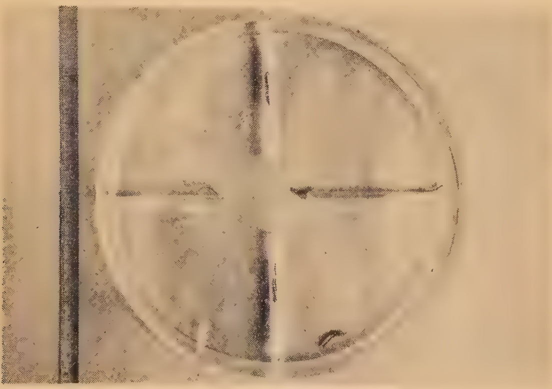


FIG. 1. Odor Trap Apparatus.

The extracting procedure from rice plants was established by a adopting the following assay method. An odor trap method consisting of four small glass tubes was used (Fig. 1).

Small pieces of absorvent cotton wool containing an appropriate amount of the sample to be tested were placed in two test tubes. The other two tubes also containing small pieces of absorbent cotton but not containing the test samples were used as controls. All the above substances were placed in a petri dish as shown in Figure 1. The test samples dissolved in methanol were placed in the tubes and allowed to dry at 90°C for 30 minutes. Ten insects grown on sterilized artificial media4) were set free in the centre of the dish and placed in the dark at 25°C for 24 hours. The insects found inside the test sample tubes and the control tubes or outside the tubes were recorded as positive (+), negative (−) and indifferent (±), respectively. Each test was repeated five times, using a total of fifty insects.

The results of fractionation and characterization of the borer attracting component obtained from rice plants showed that the substance was soluble in alcohol and ether. It was neutral and distillable at 100°–140°C, at 3 mmHg. The chromatogram with silicic acid and hexane5) showed that this substance was chromatographically polar. Hydrolysis with 2N alcoholic potash demonstrated that this substance is contained in the unsaponifiable fraction. After rechromatographing the unsaponifiable oil with silicic acid and hexane, the effluent with hexane containing 1% ethanol was distilled. The distillate obtained was about 50 mg from one Kg of air-dried rice plants.

---

1) J. W. Ingram, E. K. Bynum and L. J. Charpentier., *J. Econ. Entmol.*, **40**, 745 (1947)

2) K. Iyatomi and T. Sugino, *Plant Protection* (*Japan*), **5**, 120 (1951).

3) T. Miyake, *ibid.*, **6**, 25 (1952)

4) S. Ishii and H. Urushibara, *Bull. Nat. Inst. Agr. Sci.* (*Japan*). Ser. C, No. **4**, 109 (1954).

5) J. G. Kirchner and J. M. Miller, *Ind. and Eng. Chem.*, **44**, 318 (1952).

The threshold dosage for the test of the above distillate was found to be 1.25 microgram per test tube (Table I).

After examination of this component by chromatography and the chromatostrip method[6], it was found separable into two components, of which the component more active as a borer attractant, was shown to be an aliphatic unsaturated ketone compound by ultraviolet (max. 251mμ) and infrared spectra examination. By the decomposition of its 2,4-dinitrophenylhydrazone (m. p. 249°C) (Fig. 2) the original compound was recovered and identified by the physiological test and absorption spectra. It was named Oryzanone after the rice plant *Oryza sativa* L., from which it was isolated. The authors wish to thank Prof. Y. Sumiki of the University of Tokyo and Prof. T. Tamura and Prof. K. Iyatomi of Nagoya University for their constant advice and encouragement.

TABLE I.
ATTRACTANT TEST WITH ORYZANONE

No. of insects	No. of test	Dosage per tube (microgram)	Response		
			+	−	±
50	5	500	19	4	27
50	5	25	19	4	27
50	5	1.25	13	7	30
50	5	0.0625	4	3	43
50	5	0.0031	2	3	45
50	5	0.0001	4	3	43

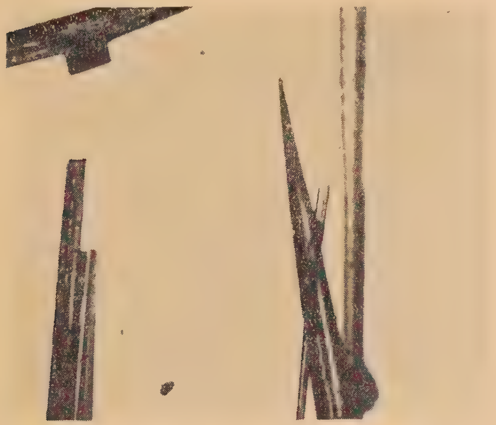


FIG. 2. 2,4-dinitrophenylhydrazone of Oryzanone.

Katsura MUNAKATA
Tetsuo SAITO
Shigeru OGAWA
Shoziro ISHII*

Faculty of Agriculture,
Nagoya University,
Anjō, Japan

Received October 17, 1958

---

[6] J. M. Miller and J. G. Kirchner, *Anal. Chem.*, **24**, 1480 (1952).

* *National Institute of Agricultural Science. Nishigahara, Tokyo, Japan.*

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 1, p. 65~67, 1959]

Hydroxyaspergillic Acid as Growth Inhibitant against Hiochi-bacteria

(Studies on Growth Inhibiton of Hiochi-bacteria, Specific Saprophytes of Saké. Part 4)

Sir:

The authors searched for antibiotics active against hiochi-bacteria using the broth filtrates of the shaking cultures among 314 strains of *Aspergillus oryzae*. The culture filtrates of 18 strains showed inhibition against the growth of hiochi-lactobacillus H-34 and true hiochi-bacillus H-1[1]. Recently, we have succeeded in isolating an antibiotic, as pale yellow crystals of m. p. 152°C from culture filtrates of these strains. This communication deals with this antibiotic.

---

[1] K. Kitahara, T. Kaneko and O. Goto, *J. Gener. Appl. Microb.*, **3**, 102, 111 (1957).

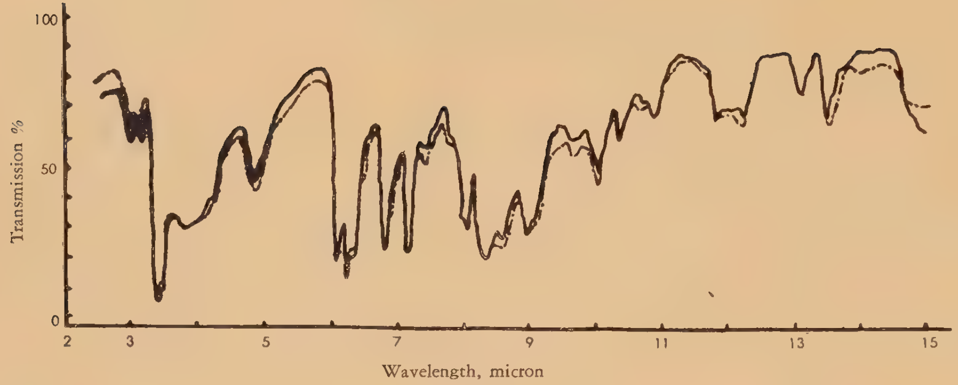


FIG. 1.

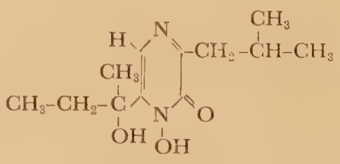
——: Prepared by authors —·—: Prepared by Dr. Dutcher

The fungi (*Asp. oryzae*, A-0-4) was shake-cultured for 7 days at 27°C aerobically in a medium containing 5% glucose, 0.5% KH_2PO_4, 0.25% $CaHPO_4$, 0.25% $MgSO_4 \cdot 7H_2O$, and 2% polypeptone. The culture filtrate was adjusted to pH 2.5 and treated with active carbon. The antibiotic was absorbed on carbon, and eluted in 60–80% acetone. After removing the solvent *in vacuo*, the rich water was acidified to pH 2.5 and extracted by ether, ethyl acetate and chloroform. The solvent layers being concentrated to syrup *in vacuo*, a crude preparation of the antibiotic was obtained. After several recrystallization in 50% ethyl alcohol or *n*-hexane, the melting point was founded to be constant at 152°C (subl.). The yield of the crude crystal from 1L. of the filtrate was 300–500 mg.

This antibiotic is obtained as pale yellow needle having a weak acidic character, and easily soluble in alcohol, ethyl acetate, chloroform, acetone, benzene, glacial acetic acid, slightly soluble in ether, *n*-hexane, but insoluble practically in water, petroleum ether. *Anal.* Found: C, 59.93; H, 7.94; N, 11.51; O, 21.22%, M.W. 276 (Rast method), 259 (titration method). Calcd. for $C_{12}H_{20}N_2O_3$; C, 60.00; H, 8.33; N, 11.66; O, 20.00%, M.W. 240. $[\alpha]^{28}_{D} = +35.85$ (ethyl alcohol C=0.3). Ultraviolet absorption in methyl alcohol, $\lambda_{max.}$ 330 mμ, $e=7500$, $\lambda_{max.}$ 232 mμ, $e=7900$, $pKa=5.15$.

Ninhydrin reaction and ferric chloride reaction were both positive. Although the Fehling reaction was negative, the redox reaction with Tollen's reagent was positive. Primary amino group could not be observed by the micro gasmetric method of Van Slyke.

Taking the above-mentioned results into account, it seems likely that the crystalline antibiotic is identical with hydroxyaspergillic acid (I), which had formerly been isolated from the culture filtrate of *Asp. flavus* by Menzel et al.[2] and established its structure by Dutcher[3], and thus, the identity was confirmed by the mixed melting point and accordance of infrared spectra (Fig. 1) with the sample of hydroxyaspergillic acid kindly supplied by Dr. Dutcher.



(I)

Biological activity; the minimum concentration for complete inhibition of growth in a diluted Saké peptone medium (composed of

---

[2] A. E. O. Menzel, O. Wintersteiner and G. Rake, *J. Bact.*, **46**, 109 (1943).

[3] J. D. Dutcher, *J. Biol. Chem.*, **232**, 785 (1958).

70 parts Saké, 30 parts water and 1 part polypeptone) against true hiochi-bacilli (*Lactobacillus homohiochii* H-42 and *Lactobacillus heterohiochii* H-1) was 10 γ/ml, and that against hiochi-lactobacilli (H-7, H-34) 20 γ/ml. However it showed almost no inhibition to the growth of *Lactobacillus saké* and *Leuconostoc mesenteroides* which were useful in Saké-brewing. At any concentration it did not inhibit the growth of *Bac. subtilis*, *E. coli*, *Staph. aureus*, *Proteus bulgalis*, *Salmonella tim*, *Mycobact.* 607, *Pen. chrysogenum*, *Asp. niger*, *Can. albicans*, *S. cereviciae*, *T. utilis*, *Corynebact. xerosis.*

Toxicity; L_D 50 for mice administered intravenously was 100mg per Kg. of body weight.

It may generally be said that many of the strains of *Asp. oryzae*, more or less produce hydroxyaspergillic acid in the shaking culture, but their activity against hiochi-bacteria was competitively inhibited by the presence of peptides.

We wish to express our sincerest thanks to Prof. Y. Sumiki of Univ. of Tokyo for his guidance and encouragement throughout the course of this work and to Emeritus Prof. K. Sakaguchi for his sound advice and also to Dr. J. D. Dutcher of the Squibb Institute for Medical Research for his kind gift of the sample used here.

Seiji NAKAMURA
Teruo SHIRO
Yamamura Saké-Brewing Laboratory,
Uozaki, Higashinada, Kobe, Japan

Received October 27, 1958

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 1, p. 67~68, 1959]

Isolation of Laminaribiose from Reversion Products of D-Glucose[1]

Sir:

It has long been known that D-glucose undergoes condensation and forms reversion products when treated by mineral acids. In recent years, by using chromatography, isomaltose[2,3], gentiobiose[2,3], maltose[3,4], cellobiose[3], nigerose[3], sophorose[3], α,α-trehalose[5], β,β-trehalose[3] and 5-*O*-β-D-glucopyranosyl-D-glucofuranose[5] have been isolated as their crystalline derivatives from acid reversion mixtures of D-glucose. Namely, eight of the eleven theoretically possible D-glucose disaccharides of pyranosidic structure have been identified. From these results, it seems that the remaining glucopyranose disaccharides must be present in these acid-catalyzed reversion mixtures.

We have been able to isolate laminaribiose as its crystalline octaacetate from reversion products obtained by the action of cation exchange resin and mineral acid on D-glucose. Cation exchange resins have recently been used as insoluble acid catalysts for the hydrolysis of disaccharides and polysaccharides[6,7].

(1) A mixture of D-glucose (50.0 g), Amberlite IR-120 (H form, 50 g) and water (200 ml) was heated with stirring in a boiling water-bath for three hours. After filtering the resin, the solution was placed on a carbon-Celite column and developed with water and 5, 15 and 25% ethanol as succesive eluting solvents.

---

[1] The first part of this paper was presented at the 183th Local Meeting of the Agricultural Chemical Society of Japan held in Tokyo, April 26, 1958.

[2] A. Thompson, M. L. Wolfrom and E. J. Quinn, *J. Am. Chem. Soc.*, **75**, 3003 (1953).

[3] A. Thompson, K. Anno, M. L. Wolfrom and M. Inatome, *J. Am. Chem. Soc.*, **76**, 1309 (1954).

[4] W. R. Fetzer, E. K. Crosby, C. E. Engel and L. C. Kirst, *Ind. Eng. Chem.*, **45**, 1075 (1953).

[5] J. C. Sowden and A. S. Spriggs, *J. Am. Chem. Soc.*, **78**, 2503 (1956).

[6] W. H. Wadman, *J. Chem. Soc.*, **1952**, 3051.

[7] R. E. Glegg and D. Einbinder, *Anal. Chem.*, **26**, 1365 (1954).

The sirup from the 15% ethanol effluent was rechromatographed on a carbon-Celite column with 5, 8 and 15% ethanol. The latter 15% effluent was evaporated under reduced pressure to a sirup which was acetylated with sodium acetate and acetic anhydride. By Magnesol-Celite chromatography using benzene-tert.-butanol as developer, laminaribiose octaacetate was separated from cellobiose octaacetate and isolated in the crystalline form; yield 70 mg, m. p. 160–161° undepressed on admixture with a known sample of β-laminaribiose octaacetate[8], $[\alpha]_D^{13}$ $-27.7°$ (c 1.8, $CHCl_3$). The infra-red spectrum of this material was also identical with that of the sample.

From other ethanol fractions, the following disaccharides were isolated as their crystalline octaacetates: isomaltose, maltose, sophorose, nigerose and gentiobiose. Isomaltose and gentiobiose predominated in resin-catalyzed reversion mixtures as they did in the case of acid-catalyzed mixtures[3].

(2) A solution of D-glucose (50.0 g) in 0.082 N hydrochloric acid (150 g) was heated in a boiling water-bath for ten hours[3]. The reaction mixture was neutralized with dilute sodium hydroxide and was chromatographed on a carbon-Celite column with water and 5, 15 and 25% ethanol. The effluents were concentrated separately under reduced pressure to sirups and the sugars in each fraction were examined by paper chromatography and paper electrophoresis. The 15% ethanol fraction containing laminaribiose was acetylated and chromatographed on a Magnesol-Celite column. Crystalline β-laminaribiose octaacetate (60 mg) was thus isolated and identified by melting point, mixed melting point and optical rotation.

Experimental details will be published elsewhere.

Kimiko ANNO
Nobuko SENO
Eiko NAKAMURA
Hatsuho SAITO
Reiko HOSHI

*Department of Chemistry,
Faculty of Science,
Ochanomizu University, Tokyo*

Received October 28, 1958

---

[8] Kindly supplied by Dr. K. Aso, Tohoku University.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 1, p. 68~70, 1959]

Reactivation of Bacterial α-Amylase Inactivated with Ethylenediamine Tetraacetate

Sir:

It has been reported by many workers that α-amylases isolated from various sources increase their stability by the addition of calcium salts while the complete removal of calcium from the enzyme solution, irreversibly results in loss of activity. It has also been reported that most of the crystalline α-amylases so far obtained contain calcium, thus leading to the publication of several papers on the role of calcium in the enzymes.

However, Fischer and Haselbach[1] have published a paper that the inactivation of malt α-amylase induced by calcium catchers such as ethylenediamine tetraacetate (EDTA) is essentially reversible. Recently, Stein[2] and Stein and Fischer[3] have reported that amylase preparation which had been purified in the presence of diisopropyl fluorophosphate (DFP), a protease inhibitor, is quite resistant towards EDTA.

---

[1] E. H. Fischer and C. H. Haselbach, *Helv. chim. acta*, **34**, 325 (1951).

[2] E. A. Stein, *Fed. Proc.* (*U. S. A.*), **16**, No. 1, March (1957).

[3] E. A. Stein and E. H. Fischer, *J. Biol. Chem.*, **232**, 867 (1958), also by private communications.

These papers strongly attracted the attention of the present authors who considered calcium to be essential for maintainance of the enzymatic nature of bacterial α-amylase[4], and thus, an experiment to confirm the fact referred above was undertaken following the method of Stein and Fischer. The result obtained was quite similar to that reported by those authors. However, in our experiments, an important phenomenon was observed, namely, the bacterial α-amylase treated with EDTA, in fact, has no enzyme activity but, by the addition of certain metal salts, recovers its activity either completely or partially, according to the kind of metal salt added. This fact may clearly indicate that bacterial α-amylase is active only in the presence of certain kinds of metals.

One gram of crystalline bacterial α-amylase (*Bac. subtilis* var. *amyloliquefaciens* Fukumoto) obtained by the method of one of the authors[5] was suspended in 40 ml of distilled water and dissolved by adding N/5 sodium hydroxide solution gradually so that the pH did not exceed 10.4. The enzyme solution was dialyzed against distilled water until the pH fell to 6.6. Then, to the solution (66 ml), 5 mg of "Potato protease inhibitor" obtained by the method of Matsushima[6], 3.5 ml of M/2 phosphate buffer solution (pH 6.6) and 0.3 ml of 0.7M DFP solution in isopropanol were added in the above order and the solution was incubated at 25°C. After five days, 10 ml of M/15 EDTA (pH 6.6, ammonium salt prepared from EDTA-4H) was added and the enzyme solution was further incubated for three days at the same temperature.

The enzyme thus treated was very sensitive to pH and was stable only in the range of pH 5.8 to 7.4, indicating a sharp contrast to the original crystalline amylase which was quite stable in the pH range of 5.0 to 10.6. On the other hand, the overall yield of enzyme activity was almost complete when the activity was determined by immediately using commercial soluble starch which contained a trace amount of calcium. In the assay of enzyme activity, the treated amylase was diluted one thousand times with distilled water and 0.5 ml of the diluted enzyme was added to a mixture of 5 ml of 2% soluble starch, 2 ml of M/20 acetic acid-ammonium acetate buffer (pH 6.2) and 2.5 ml of distilled water (sometimes, salt solutions) at 37°C. At suitable time-intervals, 0.5 ml aliquots of the reaction mixture were taken into 0.5 ml of N/1000 iodine solution and the reaction time required for the iodine-reaction colour to turn red was measured (The colour developed was compared with a standard.). However, when the assay was carried out using soluble starch dissolved in EDTA

TABLE I. EFFECTS OF VARIOUS ALKALINE AND ALKALINE EARTH METAL SALTS ON BACTERIAL α-AMYLASE INACTIVATED WITH EDTA

(The experimental condition was the same as that in Fig. 1, only that the salt solutions added were 0.5 ml. After incubating the enzyme-substrate mixture (9.5 ml) containing ammonium-EDTA for 20 min., salt solutions of which pHs were adjusted with acetic acid or ammonia, were added and the reactivation degrees were compared by the assay method.)

Salt* added	None	Li	Na					Na**	K	Be	Mg	Ca	Sr	Ba
Final conc., M	—	1/50	1/20	1/40	1/50	1/80	1/160	1/80	1/50	1/60	1/60	1/60	1/60	1/60
Time to the end point, min.	*** ∞	*** ∞	24	40	48	80	245	78	**** ∞	31	13	8	11	14

*, acetate.
**, sodium-EDTA.
After 18 hrs., *** blue, **** violet, respectively.

---

4) T. Yamamoto, This Bulletin, **20**, 188 (1956), J. Fukumoto, T. Yamamoto and et al., *Symposia Enz. Chem.* (*Japan*), **14**, 193 (1958).
5) T. Yamamoto, This Bulletin, **19**, 121 (1955).
6) K. Matsushima, *Nippon Nogei-Kagaku Kaishi*, **31**, 38 (1957), also by a private communication.

solution (ammonium salt, M/60, pH 6.0), in order to sequester the contaminating calcium, no enzyme activity was found in the enzyme solution[7], as seen in Table I. But, as shown in Fig. 1, the addition of calcium salt to the enzyme-substrate mixture brought a sudden appearance of enzyme activity and enzyme reaction proceeded in almost similar velocity to that found in the activity assay using soluble starch without treatment with EDTA.

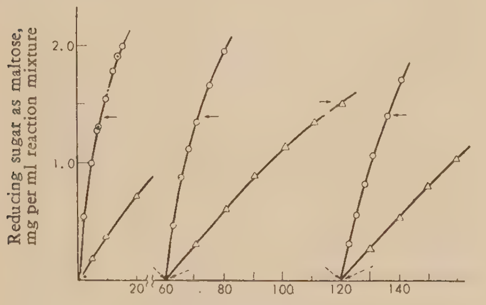


FIG. 1. Reactivation of Bacterial α-Amylase Inactivated with EDTA.

The mixtute of 5 ml of 2% soluble starch in M/60 EDTA (ammonium salt, pH 6.0), 2 ml of M/20 acetic acid-ammonium acetate buffer (pH 6.2), 2.5 ml of water and 0.5 ml of amylase was incubated at 37°C and, at the points indicated by ↘, 0.2 ml of a molar solution of Ca-acetate (—○—) or Na-acetate (—△—) was added. →, red point of iodine reaction; —◉—, sugar formation found when using soluble starch without EDTA-treatment.

The reappearance of enzyme activity was observed over several hours of incubation but, under the condition (37°C), the degree of reactivation decreased to about one half in a six-hour incubation, owing to the instability of the treated enzyme. The reactivation of the enzyme was also achieved by other alkaline earth metal salts, but all their effects were less than that of calcium salt, as seen in Table I. Of the alkaline metal salts tested, only the effect of sodium was distinct while those of potassium and lithium were extremely small or entirely negative. The effect of sodium salt appeared to be a function of concentration of the salt (Table I). The influences of heavy metal salts have not yet been made clear. It is desirable to use the soluble starch freed from calcium as the substrate in this experiment, otherwise the calcium being chelated with EDTA in the reaction mixture will be freed and give an effect to the enzyme, by the addition of heavy metal salts that have a larger chelation constant with EDTA than calcium.

The results in the present paper may be satisfactory to demonstrate that bacterial α-amylase is inactivated with EDTA and re-activated with certain alkaline- and alkaline earth metal salts, and may lead to the conclusion that bacterial α-amylase does require certain kinds of metals, in order to display the enzymatic action. The fact that the degree of reactivation of the EDTA-inactivated enzyme significantly differs according to the kind of metal salt added, seems to provide a clue for elucidating how the metals share in the enzyme action. Also, the fact in the present paper, together with the findings of Stein and Fischer, seem to have proved that the natural existence of calcium in bacterial α-amylase contributes not only to the stabilization of the enzyme, but also to the display of enzyme action.

The authors wish to express their sincere thanks to Prof. Izue Yamasaki of Kyushu University, for his instructive suggestion and encouragement in this work. They also wish to express their cordial thanks to Drs. Edmond H. Fischer and Eric A. Stein of Washington University, Seattle, U. S. A., for their helpful advice and discussion.

Takehiko YAMAMOTO
Juichiro FUKUMOTO

The Institute of Polytechnics,
Osaka City University

Received October 13, 1958

---

[7] When the non-treated enzyme, even its extremely dilute solution, was added to soluble starch dissolved in EDTA solution, as in the above experiment, enzyme reaction occurred and proceeded, to certain extents, almost similarly to that found in case of using soluble starch without EDTA-treatment.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 1, p. 71, 1959]

Decomposition and Formation of Oxalate in Fish Skeletal Muscle

Sir:

Recently, the authors found that crude enzyme solution prepared from carp skeletal muscle in summer by adsorption with $Ca_3(PO_4)_2$ and by elution with phosphate buffer pH 7.4, showed strong decomposing activity of oxalate, but scarcely did malonate.

However, the same enzyme preparation obtained in autumn indicated marked activity for malonate decarboxylase as previously reported[1].

In case of marine fish, the same tendency was observed as shown in the table I. Two enzymes were entirely different as described below. Optimum pH of oxalate decomposing enzyme was ca. pH 4.0 and that of malonate decomposing one was ca. pH 5.0. The former was destroyed at 60°C and the latter was done at 85°C.

Furthermore, it was especially confirmed that enzyme preparation in summer remarkably revealed malonate decarboxylating activity after it was dialysed, and this action was again hindered by the addition of boiled water extract of fish muscle.

The formation of oxalate in vitro was also observed, as shown in the table II. 50 mg of each org. acid Ca salt was added to 200 cc M/15. phosphate buffer extract, prepared from each 100 g meat of marine fish (*Oplegnathus fascinatus*), refrigerated immediately after beheading. After being kept in test tubes at 30°C for 20 minutes, mixtures were deproteinized with trichloroacetic acid and neutralized with $Ca(OH)_2$. Acetic acid soluble salts being discarded, Ca oxalate was decomposed with 1N H_2SO_4. Oxalic acid was colorimetrically determined by adding 0.05% Indol solution in conc. H_2SO_4, according to the method of J. Bergerman et al.[2].

Judging from the above result, oxalate seems to be a dominant intermediate in succinate metabolism of fish skeletal muscle under active state, but C_4 dicarboxylic acids or Krebs cycle might come to appear in a close-up with fading away of oxalate decomposing activity under special circumstances.

Kisaku YAMADA
Taneko SUZUKI
Mizuho TANAKA

Seikai Regional Fisheries,
Research Laboratory,
Nagasaki, Japan

Received November 12, 1958

TABLE I. ACTIVITY OF OXALIC ACID AND MALONIC ACID DECOMPOSING ENZYME IN SUMMER SEASON

$$Q_{CO_2}=\frac{CO_2\ \text{output (cmm)}}{\text{dry matter (mg)}\times\text{hour}}$$

Kind of Fish		*Sardinia melanosticta*	*Scomber japonicus*	*Zeus japonicus*	*Argirosomus nibe*	*Lepidotrigla güntheri*	*Batoidei* sp.	*Trachurus japonicus*
Month		August	June	August	June	August	August	June
Q_{CO_2}	Oxalate	6.6	5.7	10.6	3.7	2.2	5.7	3.1
	Malonate	0.0	0.0	0.0	0.8	0.0	0.3	—

TABLE II. FORMATION OF OXALIC ACID BY THE EXTRACT OF FISH SKELETAL MUSCLE

Substrate		Citrate	Tartarate	Succinate	Fumarate	Malate	Oxalacetate	Acetate	Formate	Glyoxylate
Oxalic acid found	pH 5.8	trace	0	1.6 (mg)	0	trace	0	0.9 (mg)	0	0
	pH 7.0	trace	0	0.7	0	trace	0	1.0	trace	0

---

[1] K. Yamada and T. Suzuki, *J. Agr. Chem. Soc. Japan*, **25**, 372 (1952).

[2] J. Bergerman and J. S. Elliot, *Anal. Chem.*, **27**, 1014 (1955).

Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI
(in Japanese)
Vol. 32, No. 11 (1958)

Studies on the Classification of *Streptomyces griseus* Group. Part II. Classification by Cultural Properties. (p. *817~823*)

By Yujiro HARADA, Shigeo KUBO and Shiro TANAKA

(*Kyowa Fermentation Ind. & Co., Ltd.*)

1. On the previous paper authors had reported the morphological classification of *Streptomyces griseus* group. On this paper, authors have reported the classification by cultural properties.

2. The description of the color of growth should be done on the washed mycelium of submerged broth. For example, by this method, the color of growth of *Streptomyces olivaceus* which was described almost black was not black but cream yellow as like as *Streptomyces griseus*.

3. According to authors experimental data, the color of aerial hyphae of *Streptomyces griseus* was varied from water green to grayish or pinkish white even on the same cultural medium and other same conditions because of the variety of the source of isolation, so authors considered that the description of the color of aerial hyphae should not be important key on the classification.

4. Also, the soluble pigment, physiological properties were varied quantitatively and should not important key.

5. It was reasonable to conclude that the description of the cultural properties should be written in wide scope on the classification of *Streptomyces griseus* group.

6. The cultural properties of *Streptomyces olivaceus* were very similar to that of *Streptomyces griseus* except for the color of aerial hyphae as same as morphological specification and authors should like to notice that *Streptomyces olivaceus* may be classified in *Streptomyces griseus* group.

Studies on the Relations among Yeast, *Rhizopus* and *Lactobacilli*. Part I. The Influence of Yeasts and *Rhizopus* on the Isolated *Lactobacilli*. (p. *824~828*)

By Shigeru NEMOTO and Yoshio IRIE

(*Godo-Shusei Inc.*)

The authors studied on the relations among Yeast, *Rhizopus* and *Lactobacilli* in order to elucidate the mechanisms of contamination by *Lactobacilli* in alcoholic fermentation by the amylo process.

In case of using liquid media of cereal materials, we discovered the fact that the *Lactobacilli* produced almost no acids if they were cultivated with *Rhizopus*, but we noticed much more acids produced in adding yeast with the advance of its propagation.

Then, incubating in the Henneberg's solution including glucose as carbohydrate source, *Lactobacilli* could not grow by itself, but could develop soon after *Rhizopus* had grown and increased with the progress of the propagation of yeast.

Furthermore, these facts were also confirmed in the case of solid media.

Carbohydrates in the Fruit of Date-palm. (p. *829~832*)

By Takashi MIZUNO and Yachiyo MIKAMI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Shizuoka University, Iwata*)

Free sugars fermented by a certain yeast, amounting to 50% for dry basis in the fruit of Date-palm (*Phoenix dactylifera* L.), were mainly composed of glucose and fructose (the molecular ratio was 1.7:1.0), containing a little amount of saccharose too.

Nine polysaccharides were isolated out of Jujube fruit by the fractionation with various solvents: cold and hot water, hot 0.5% ammonium oxalate solution, cold and hot 5% NaOH, cold 20% NaOH, successively.

The properties of these polysaccharides would be found in the table.

And it was made clear that saccharose 3.82%, glucose 1.68%, fructose 1.53%, raffinose 0.30% and stachyose 0.22% for dry basis exist as free sugars in the seed of Jujube.

Various polysaccharides were isolated from the Jujube seed with the same fractionation as mentioned above, and chemical and physical properties of their polysaccharides were investigated.

The constructive sugar of every polysaccharide, except that of hot water-soluble polysaccharide, was mainly mannose; and also other sugars of glucose, galactose, fructose, galacturonic acid, arabinose and xylose were found to be present.

Polysaccharides	Solvents	Yields (% for dry fruits)	Constructive sugars								Acetyl derivatives	
			Rh	Xy	Ar	Ri	Ga	Gl	Fr	Gall	$[\alpha]_D$ (in $CHCl_3$)	content of CH_3CO (%)
1	cold H_2O	1.24	+	−	++	−	+++	−	+	++	+ 23.9°	44.2
2	hot H_2O	0.31	+	−	+++	−	+++	−	+	±	+ 28.2°	44.7
3	hot 0.5% NH_4-Oxalate	0.08	±	−	++	−	++	−	−	+++	−105.3°	43.4
4	cold 5% NaOH (ppt. with AcOH)	1.13	−	−	+++	±	++	−	+	−	− 18.8°	43.9
5	cold 5% NaOH (ppt. with EtOH)	0.35	−	−	+++	±	++	−	±	−	− 72.6°	42.8
6	hot 5% NaOH (ppt. with AcOH)	0.52	−	−	++++	±	−	−	−	−	− 42.5°	43.3
7	hot 5% NaOH (ppt. with EtOH)	0.02	−	+++	−	±	−	−	−	−	− 36.7°	43.5
9	cold 20% NaOH (ppt. with EtOH)	0.16	−	++++	−	±	−	++	−	−	+ 11.4°	42.6
10	cold 20% NaOH (insoluble)	2.14	−	−	−	−	−	+++	−	−	+ 49.9°	43.4

Remarks:

Rh: rhamnose, Xy: xylose, Ar: arabinose, Ri: ribose,

Ga: galactose, Gl: glucose, Fr: fructose, Gall: galacturonic acid.

(Note) Polysaccharide 8 was not isolated from the fruit.

Studies on the Variation of Contents of Free Amino Acids during the Course of Germination of Leguminous Seed. Part II. Experiments on the Leguminous Seeds Harvested in Autumn.

(p. *833～837*)

By Ayako MATSUSHITA

(*Department of Nutrition, Kumamoto Women's University*)

The variation of contents of free amino acids during the course of germination of Leguminous seeds harvested in autumn, has now been investigated in detail by the combined use of ion exchange chromatography and paper partition chromatography.

Total free amino acids adsorbed on a column of Amberlite-IR-120 (H) (2×250 cm) were thoroughly eluted with aqueous ammonia (5%), and the combined eluate was taken to dryness in vacuo, after which the residue was dissolved in 10 ml of distilled water.

A volume of 0.001ml of this solution was chromatographed on a sheet of paper by the two-dimensional, ascending development method, using 0.1% acetonic solution of ninhydrin as a chromogenic spray reagent.

Each spot of chromatograms of amino acids was cut off, and to this was added 2 ml of 1% of aqueous solution of ninhydrin and 1ml of 10% aqueous solution of pyridine, and the solution was boiled on a water-bath for 25 min.

Each of the colored eluates was subjected to spectrophotometrical determination at 570 mμ (in the case of proline and hydroxy proline at 440 m).

The results of experiments were tabulated in Table 2 (A), (B).

Chromic Oxide Indicator Method for Digestibility Studies with Swine. (I) Variation in Chromic Oxide Excretion.

(p. *838～842*)

By Shikao ISHIKAWA

(*Faculty of Agriculture, Mie University*)

Studies have made of the effect of the method of Cr_2O_3 administration on the regularity of its excretion. The index material was administrated at 1% of the feed intake in each test.

1) The indicator was not excreted uniformly in proportion to the feces dry matter until 2.0–2.2 days elapsed after its administration. Securely it may be 3 days for the preliminary period of Cr_2O_3 feeding.

2) No significant differences were found among the rates of Cr_2O_3 obtained with the sample feces in any time or day, when the indicator was throughly mixed and ingested in fixed ratio to the feed at each regular feeding three times daily. The results were similar to those, obtained in restricted feeding where the experimental subjects received the indicator mixed with a portion of each feed. And so the at-feeding method was presumed to be most adequate for the indicator method with swine.

3) The indicator was uniformly distributed in feces obtained in the at-feeding. So the representative sample for Cr_2O_3 analysis must be taken from any part of feces mass.

4) When one day's portion of Cr_2O_3 was ingested once or twice in equal parts daily, the statistical analysis showed significant differences among the rates of Cr_2O_3 obtained with the sample feces at different times.

5) In the at-feeding, the relative standard error of the contents of Cr_2O_3 in feces dry matter was of same degree to that of organic matter or crude protein.

Ultraviolet Spectrophotometric Micromethod for Determination of Biuret as Manganese Complexes. (p. *842~847*)

By Yasuto WATANABE

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

A micromethod is given for the determination of biuret in biological materials.

Sample fluids are chromatographed on filter paper with water saturated *n*-butanol. The paper is cut at the position corresponding to the chromatogram of biuret located on the index paper by spraying with *p*-dimethylaminobenzaldehyde and immersed in the solution prepared by mixing 4 ml. 2N NaOH, 2 ml. 10 mg% $MnSO_4$, and 4 ml. water. After beeing left for 10 minutes with occasional stirring, the solution is centrifuged for 10 minutes at 3000 r.p.m. The supernatant is measured for its extinction at the wave-length of 225 mμ using the solution prepared from the paper chromatographed without applying biuret as blank.

By the postulated method, biuret can be determined at the amount of 5 to 100γ with the standard error of ±3%.

Using this method, it was found that biuret given orally to the adult rat was detected in urine at 5 minutes after administration, and excreted almost completely after 2 to 3 hours.

Studies on Flavin. Part I. Distribution and Forms of Flavin in Animal and Plant Tissues. (p. *847~851*)

By Hisateru MITSUDA, Fumio KAWAI and Toshio MIYOSHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University*)

The total riboflavin contents and its three forms in several animal and plant materials were determined by the fluorometric method. In rat tissues, as well be known, FAD was 70–80 per cent, FMN 15–30 per cent of the total contents, while free riboflavin was few. In egg and cow's milk, however, free riboflavin was about 50 per cent of the total amounts. These relations were also observed in plant materials. For example, radish or carrot root was similar to the rat tissues and wheat powder or polished rice to egg and cow's milk in the ratio of each forms. A considerable amount of free riboflavin was always found in plant materials, especially in green leaves, a half of the total contents was free riboflavin form. But the ratio of two flavin nucleotides in green leaves seemed to correspond to that of rat kidney. The distribution in cell fractions of rat liver and spinach leaf was also estimated.

A Microdetermination of Maleic Hydrazide by Reduction-cleavage in Acid Medium and the Hydrazide Absorption by Mung Bean. (p. *851~855*)

By Yasutoyo NAGAI

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

A method for microdetermination of maleic hydrazide residues in plant tissue was devised. The method consists of the reduction-hydrolysis of the hydrazide with zinc in acid medium of 4N sulfuric acid at 100° and colorimetry of the hydrazine liberated in the hydrolysate. The presence of carbonyl compounds such as fructose and ascorbate in the hydrolysis medium gave a lower result. The time-course of the hydrazide absorption by mung bean was measured by use of the method.

Butanol Fermentation. Part XXIX. Fermentation in High Concentration of Sugar (2). Sorption Method by Use of Active Carbon (3). Recovery of Solvents from Carbon. (p. *855~858*)

By Izue YAMASAKI, Motoyoshi HONGO, Kyosuke AKABOSHI and Ken NAGATA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

Experimental data of recovery of solvents from carbon in order to use sorption method by use of active carbon to butanol fermentation in high concentration of sugar was presented as follows.

1) 98% of butanol and 99% of acetone could be recovered from active carbon which sorped solvents by means of eluting as much as possible at 150°C and then steaming.

2) Active carbon took off solvents could be used repeatedly without refreshing. Ratio of sorption of active carbon was a little reduced by repeated sorption using comparative soft carbon, but hardly reduced by use of hard carbon.

Addendum ;

Sorption method of evaporating solvents by means of aeration after fermentation in high concentration of sugar proceeded was used for the recovery of solvents to *Clostridium acetobutylicum* 314 and 179, but its effect was less than that of sorption method which is above described 1).

Fermentation broth was redox-potentially anaerobic in spite of aeration.

Chemical Studies on the Autolysis of Meats. Part IX. On Combinations of Muscular Proteins with Anionic and Cationic Dyes during Aging of Meats. (p. *858~861*)

By Masao FUJIMAKI and Nobuhiko ARAKAWA

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

Combining changes of actin and actomyosin with dye ions during of meats were recognized by equilibrium dialysis method.

The combining capacity of actomyosin-S with dye ion was larger than that of actomyosin-L.

Different from most globular proteins, the combining capacity of actomyosin with methylene blue was larger than that with methyl orange.

The combining capacity of actomyosin-S with dye ion was maximum at two days after slaughter. Especially the combination with a cationic dye, methylene blue was found to be prominent.

The relation between this change and the water-combining capacity was considered.

During aging of meats, the combining capacity of actomyosin-L and actin with methylene blue decreased.

The combining capacity of actomyosin with dye ion seemed to be different by various meats.

Studies on Esterases from Seeds. Part I. Estimation of Esterase-Activity (1). (p. *861～867*)

By Tetsujiro OBARA and Yasokichi OGASAWARA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University of Education*)

We have examined here the method of estimation on esterase activity by the *p*-nitrophenol (*p*-NP) method in order to determine a very small quantity of esterase contained in seeds more quickly and exactly than the methods used before.

In the *p*-NP method, a quantity of *p*-NP liberated from *p*-nitrophenyl acetic acid (*p*-NPA) is measured by the ultra violet absorption spectra. In this experiments, the stability of *p*-NPA dissolved in various buffer solutions was estimated by the ultra violet absorption spectra.

The following results were obtained from the experiments by the method mentioned above:

1) *p*-NP dissolved in various buffer solutions was extremely stable.

2) The method which estimates a quantity of *p*-NP in the solutions at pH values below 4.68 by 315 mμ wave length indicated most effective result.

3) *p*-NPA dissolved in buffer solutions at pH values above 8.12 was very untable.

Consequently, we could not use *p*-NPA as a substrate under this condition. But *p*-NPA in the solutions at pH values below 4.68 was very stable.

4) We could use *p*-NPA as a substrate, in the case of enzyme reaction not lasting for long time in solutions at pH values near 7.0.

5) Aqueous solution of *p*-NPA was not suitable as the stock solution, but methanol solution was very stable.

Studies on Esterases from Seeds. Part II. Preparation of Esterase from Rice-Embryo (1). (p. *867～871*)

By Tetsujiro OBARA and Yasokichi OGASAWARA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University of Education*)

We have prepared the esterase from rice-embryo to clarify characteristic of the esterase, and trying to make more effective use of fats in rice-embryo.

In this experiments, we have carried out the preparation of the esterase from rice-embryo based on absorption purification used lead phosphate, examining by paper electrophoresis.

The experiments mentioned above resulted as follows:

1) Among the experiments done by the elution method on esterase from rice-embryo, the elution method by $0.025\text{N-NH}_4\text{OH}$ solution was the most excellent one.

2) We have prepared the embryo-esterase solution having specific activity of 7.5 times of the first crude solution, by the absorption purification that added lead phosphate to such extract by using NH_4OH.

3) Among the proteins contained in such solution, the esterase was the protein which is movable by electrophoresis.

4) The esterase protein contained in rice-embryo was extremely small quantity.

5) The embryo-esterase was completely inactivated, after the treatment by dialysis and electrophoresis. Consequently, the esterase was presumed to be a enzyme activated by a certain substance, not by a protein.

6) The embryo-esterase was not inactivated by the lyophilization.

Studies on the Aerobic Sugar Metabolism of Yeast. Part III. Selection of Gluconic Acid Producing Yeast. (p. *872～876*)

By Shoichi TAKAO

(*Applied Mycological Laboratory, Faculty of Agriculture, Hokkaido University*)

Previously, it was reported that several strains of *Candida* sp. isolated from fruits produced a large amount of gluconic acid from glucose in the cultural solution added $CaCO_3$.

In this experiment, for the purpose of obtaining strains of which gluconic acid producing abilities are stronger than above strains, 102 strains of yeasts isolated from various fruits were tested. As a result, it was recognized that 81 strains of *Candida* sp. had that ability, and 14 strains of them gave the yields over 50% based on glucose employed for 14 days.

And then the quantities of gluconic acid produced by 6 strains which gave the yields over 60% were compared from 7 to 23 days, and every strains gave their maximal yields after 14 to 18 days and glucose was almost consumed after 14 days.

From these experiments, *Candida* sp. C 37 that was found as the best gluconic producing strain, gave such a high yield as 76.6% based on glucose employed for 14 days.

Studies on the Utilization of Ion Exchange Process in Sugar Refining. Part III. On the Color Contamination of Ion Exchange Resins.

(p. *876～882*)

By Susumu IWASHINA

(*Research Institute of Meiji Sugar Mgf. Co. Ltd., Kawasaki, Japan*)

In general a phenomenon called color contamination appears ordinarily with increase of cycles in utilizing the ion exchange resins. In the refining process of sugar in which $R\equiv NX$ type anion exchange resin is used in Cl-form this was recognized at 30–40 cycles and showed remarkable decrease in decoloziring capacity.

The sum of seven color index at respective wave lengths between 400 and 720 mμ were estimated by spectroscopic analysis, and the volume number (ml) corresponding to Bx 50 was calculated in the case of sugar liquor, whereas in the case of eluted solution by regeneration each color weight was calculated from the value that was gained by multiplying volume number to the sum of color index of the liquor diluted to a quarter of the above amount according to the fundamental experiments. Then exchanged quantity by the resin,

$$\text{Regeneration Efficiency } (\%) = \frac{\text{color weight eluted from the resin on regeneration}}{\text{color weight adsorbed by the resin}} \times 100,$$

and

$$\text{Ratio of Color Contamination } (\%) = \frac{\text{color weight remained in the resin on regeneration}}{\text{color weight in the treated liquor}} \times 100,$$

was obtained absorbed coloring substance were not eluted perfectly by NaCl solution only and the resin was colored significantly and they remained and accumulated. But in the case of two kind of the resins (Type-I, Porous resin), on examination of liquors passage up to 50 cycles, salt-splitting capacity decreased by 23.5 and 35.45% respectively and moveover increase of Fe as well as other inorganic matters were recognized in contamination of the resins.

From the results defined, the color contamination may be regarded as metal salts derived from treated liquors accumulated in the exterior and interior of net structure of the resin or organic impurities irreversibly adsorbed, and it was pointed out that the degree was controlled by chemical and physical property of cross-linking of the styrene matrix and by method of applying and its history as external factor and that the composition of contacting sugar liquor, in other words quantity and form of the contaminating matters contained in it became a factor which determined the time of obstacle due to the color contamination.

Studies on the Utilization of Ion Exchange Process in Sugar Refining. Part IV. Tendencies of Color Contamination on the Strongly Basic Anion Exchange Resins and its Affection of Inorganic Salts within Sugar Solutions. (p. *882～889*)

By Susumu IWASHINA

(*Research Institute of Meiji Sugar Mfg. Co. Ltd., Kawasaki, Japan*)

Four commercially available anion exchange resins were regenerated to Cl-form and were led to constitute single bed of the resin and clarified cuban and formosan sugar liquor which is treated with carbonation and active carbon methods as pretreatment were passed both intact (I) and after enriched its salt content with Na_2SO_4 (II).

The color contamination tendency of the resin between 1–20 cycles and the influence of salts upon it were investigated from the fluctuation of color weight calculated from color index which were obtained spectrophotometrically from the absorbancy at wave lengths of 420 and 720 mμ about the treated liquor.

The results showed that decolorization, regeneration and mutual relation between color contamination had their original cause closely in the structure of the resin matrix especially in porosity and the elution of exchangeable material adsorbed had almost reached equilibrium in (I) or (II) at 15–20, or 10–15 cycles respectively in connection with regeneration efficiency and decolorizing power. Because exchange of SO_4^{--}–Cl^-, as small molecular ion which is advantageous in diffusing into net structure of the resin in the case of (II), is preferable to coloring material aided by the strength of affinity to functional group of the resin, the uniform change are given to decolorizing power, regeneration efficiency and ratio of color contamination.

Especially this phenomena was verified by the fact that in B of Type II-Porous, on the ratio of color contamination was low and regeneration efficiency was high. Moveover 3 stage regeneration by 10% NaCl solution was examined after 20 cycles without any success in removal of contaminated substances and accordingly the imperative necessity of the restoration was recognized, as its countermeasure.

Biochemical Studies on the Blast Disease of Rice Plant. Part X. The Biochemical Classification on *Piricularia oryzae* Cavara (5). (p. *890～893*)

By Hitoshi OTSUKA*, Kinjiro TAMARI** and Nagahiro OGASAWARA**

(**Faculty of Education*, *University of Niigata*; ***Faculty of Agriculture*, *University of Niigata*)

Forty-seven strains of *Piricularia oryzae* Cavara have been tested for their ability to utilize three organic acids which related to the tricarboxylic acid cycle as a source of carbon in chemically defined medium. And it was investigated that nine strains were utilize these organic acids.

Cellulase and pectinase were also investigated.

Biochemical Studies on the Blast Disease of Rice Plant. Part X. The Biochemical Classification on *Piricularia oryzae* Cavara (6). On the Production of Nicotinic Acid by *Piricularia oryzae* Cavara. (p. *893～897*)

By Hitoshi OTSUKA*, Kinjiro TAMARI**, Nagahiro OGASAWARA** and Ryoji HONDA*

(**Faculty of Education*, *University of Niigata*; ***Faculty of Agriculture*, *University of Niigata*)

The nine of forty-seven strains *Piricularia orizae* Cavara were able to grow on thiamine free synthetic media, but other strains were not. The eight of thiamine required strains were able to grow on synthetic media added nicotinic acid instead of thiamine. And it was also investigated that *Piricularia oryzae* Cavara produces the nicotinic acid. Forty-seven strains of *Piricularia oryzae* Cavara were classified into thirteen types from various biochemical differences.

Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI
(in Japanese)
Vol. 32, No. 12 (1958)

Growth Inhibition of the Mung Bean Seedling by Maleic Hydrazide. (p. *899～903*)

By Yasutoyo NAGAI, Jun-ichi SAITO and Saburo FUNAHASHI

(*Department of Agricultural Chemistry*, *Faculty of Agriculture*, *University of Tokyo*)

Treatment of germinating mung bean with 100 p.p.m. maleic hydrazide caused a considerable inhibition of the seedling growth. The treatment resulted in depression of water absorption by roots and their elongation, of development of lateral roots and of oxygen uptake by the seedling, accompanied with the decrease in weight of dry matter of cotyledon. The retardation of the embryo growth could be seen from decrease in its dry weight and nitrogen content. The rate of increase in amounts of fructose, ascorbate and arginine became lower as compared with those of control. The mitochondrial fraction from the hydrazine-treated seedling showed the same Qo_2 value for succinate oxidation as that from the untreated. In vitro addition of maleic hydrazide to the mitochondrial preparation was without effect on its succinate oxidizing activity.

The Influence of Iron on the Production of Gluconate and 2-Ketogluconate by *P. ovalis* and *P. fluorescens*. (p. *903～907*)

By Takeshi TAKAHASHI and Hisashi KAYAMORI

(*Faculty of Agriculture*, *Tokyo University of Agriculture and Technology*)

Incubating *P. ovalis* and *P. fluorescens* in the iron deficient glucose medium, the glucose dehydrogenase is activated and the gluconate dehydrogenase is inhibited. The minimum concentration of iron on the production of 2-ketogluconate is about 0.01p.p.m.. It is not able to replace iron with other metals. The oxidation of 2-ketogluconate is not affected by the iron, so gluconate accumulates in the iron deficient medium of *P. fluorescens* producing 2-ketogluconate.

Chemical Studies on the Tanning Properties of Animal Skin. Part VI. On the Adsorption of Tannic Acid to Skin Collagen. (p. *907~913*)

By Yasushi SATO*

(*Laboratory of Agricultural Chemistry, Department of Agricultur, University of Tokyo*)

Some experiments on the reaction between skin collagen and tannic acid were carried out and some discussion were presented on the basis of their experimental results and author's previous reports.

It is concluded that adsorption of tannic acid by skin collagen is consisted of two reaction: one is the ionic reaction between dissociated amino groups of skin collagen and acidic groups or colloidal ion of tannic acid which is increased stepwise according to increase of its concentration, and the other is the non-ionic reaction between such undissociated groups as peptid chain of skin collagen and hydroxyl groups of tannic acid.

The former reaction is showed by the following experiments: the influence of adding HCl on adsorbed tannic acid, the relation between the change of pH accompanied with the change of concentration of tannic acid solution and the change of adsorbed tannic acid, etc..

The latter reaction is showed by the following experiments: the influence of adding NaOH on adsorbed tannic acid, its adsorption in organic solvent, the relation between the equilibrium concentration in adsorption and adsorbed tannic acid, the decrease of bonding water showed after the adsorption of tannic acid, the correlation between the change of surface tension in its solution and the change of adsorbed tannic acid, etc..

* present post: *Dept. of Agr., Univ. of Nagoya.*

Studies on the Mechanism of Urea-utilization by Ruminants. Part I. On the Urease in Beef Gastric Mucosa. (p. *913~917*)

By Kensuke SHIMURA, Masami SOEJIMA and Eiichi KIKUCHI

(*Laboratory of Biochemistry, Faculty of Agriculture, Tohoku University*)

Fresh beef stomachs were divided into six parts, viz., blind sac of rumen (Ia), dorsal and ventral sac of rumen (Ib), reticulum (II), omasum (III), pyrolic part of abomasum (IVa) and the major body of abomasum (IVb). Urease was extracted from the acetone-dried powder of the mucosa with twenty volumes of physiological saline solution, pH 7.0, and purified by a modified method of Sumner[1] and Dounce[2].

The total activities of urease in each part were as follows; Ia 250, Ib 500, II 0, III 0, IVa 1,100 and IVb 55,000 units per head. The one unit of the activity is defined as an amount (γ) of NH_3, which is liberated during 90 min. incubation in 0.5M urea solution (pH 7.0).

The partially purified urease from IVb was found to have an electrophoretic mobility of 9.0×10^{-5} cm/sec. volt at pH 7.8 (μ 0.144), optimum pH of 7.2, Michaelis' constant (pKm) of 1.5, appearent activation energy of 7.3–7.4 cal per mol, and was inhibited by thiol reagents such as *p*-chloromercuribenzoate, monoiodoacetamide.

These data indicate that beef stomach urease is similar to sword bean urease.

[1] J. B. Sumner, *J. Biol. Chem.*, **69**, 435 (1926).
[2] A. L. Dounce, *J. Biol. Chem.*, **140**, 307 (1941).

Studies on the Mechanism of Urea-utilization by Ruminants. Part II. On the Adaptive Formation of Urease by Rumen Microorganisms. (p. *917~921*)

By Eiichi KIKUCHI, Masami SOEJIMA, Kiyoshi SUGAWARA and Kensuke SHIMURA

(*Laboratory of Biochemistry, Faculty of Agriculture, Tohoku University*)

Three- to nine-month-old kids were used as experimental animal. Ten to thirty ml. of the rumen contents were incubated with a culture medium containing urea at 39°C (pH 6.8). After the incubations, ammonia formed and urea remained were measured, respectively. It was proved that there were two types of urease activity in the rumen; i. e., the constitutive one and the potential one. The former, which was generally weaker than the latter, varied with season and the feed given. On the other hand, the latter was shown to be an adaptive enzyme.

The rumen contents, furthermore, were fractionated by differential centrifugation. Supernatant at $8500 \times g$ had no urease activity, and the activity of potential urease was found in the fraction precipitated at $200 \times g$. The latter fraction consisted mainly of the rumen protozoa.

Studies on the Mechanism of Urea-utilization by Ruminants. Part III. The Utilization of N^{15}-

Urea in Goat Rumen. (p. *921～924*)

By Kiyoshi SUGAWARA, Masami SOEJIMA, Shōnosuke SAGISAKA and Kensuke SHIMURA

(*Laboratory of Biochemistry, Faculty of Agriculture, Tohoku University*)

A male goat, 5 months old, was fed with 1.5 g of N^{15}-urea (7.0 atom per cent excess of N). Normal feed was restricted for 5 hr. prior to the urea feeding. Rumen contents were taken at 1.4 and 7.5 hr. after the urea feeding, filtered with gauze and the filtrate was fractionated into protozoan fraction (200 × g 10 min.), bacterial fraction (6,000 × g 20 min.) and supernatant fluid by differential centrifugation.

The urea was rapidly converted to NH_4^+ in the rumen and then the NH_4^+ formed decreased with time. On the other hand, N^{15} content of protozoan and bacterial fraction were increased. That is, at 1.4 and 7.5 hr. after the urea feeding N^{15} contents of bacterial fraction was 0.08, 0.25 and 0.23 atom per cent excess N and for protozoan fraction, 0.04, 0.09 and 0.11 atom per cent excess N, respectively.

From the results described in part I, II and III of this study it was suggested that urea was converted to NH_4^+ by protozoan urease and then a large part of this NH_4^+ was taken up by bacteria and some of them by protozoa.

Studies on the Classification of *Streptomyces griseus* Group. Part III. Classification by the Utilization of Carbon and Nitrogen Compounds. (p. *924～929*)

By Yujiro HARADA, Shigeo KUBO and Shiro TANAKA

(*Tokyo Research Laboratory, Kyowa Fermentation Ind. & Co., Ltd.*)

1. On the previous paper authors had studied the classification by cultural properties. On this paper, authors have reported the classification of *Streptomyces griseus* group by carbon and nitrogen utilization spectrum.

2. According to authors' studies on the fluctuation of carbon utilization spectrum in many progeny, mutagenic process and isolation sources, the utilization spectrum of pentose and hexose was very stable key in the classification. The utilization spectrum of disaccharide tended to vary according to the variety of isolation sources. The utilization spectrum of trisaccharide and sugar alcohol may be effective key in the classification only in the case of positive utilization is occured.

3. On the classification by carbon utilization spectrum, *St. olivaceus*, *St. griseolus* and *St. griseoflavus* are the almost same species as *St. griseus*.

Studies on the Classification of *Streptomyces griseus* Group. Part IV. Classification by the Actinophage Sensitivity. (p. *929～932*)

By Yujiro HARADA and Shigeo KUBO

(*Tokyo Research Laboratory, Kyowa Fermentation Ind. & Co., Ltd.*)

1. On the previous paper authors had reported the classification by the carbon utilization spectrum, on this paper, authors have reported the classification by actinophage sensitivity.

2. According to authors' experimental data, the typical strains of *Streptomyces griseus* had severe sensitivity against six strains of the actinophages of *Streptomyces griseus* which were stocked in author's laboratory, but *Streptomyces griseus* var. *rhodochrous* SN and grisein-producing strains had no sensitivity against these actinophages. Several strains of *Streptomyces olivaceus, Streptomyces griseolus*, *Streptomyces flavovirens* and *Streptomyces scabis* had sensitivity against these actinophages.

3. Authors come to have one opinion that *Streptomyces olivaceus* may be the same as *Streptomyces griceus* according to the similarity of morphology, cultural properties, carbon utilization spectrum and actinophage sensitivity.

Studies on the Classification of *Streptomyces griseus* Group. Part V. Classification by Antagonisms. (p. *932～936*)

By Yujiro HARADA and Shiro TANAKA

(*Tokyo Research Laboratory, Kyowa Fermentation Ind. & Co., Ltd.*)

1. On the previous paper authors had reported the classification by the actinophage sensitivity, on this paper, authors have reported the classification by antagonisms.

2. According to authors' experimental data, *St. griseus* Waksman line, S-1 line, Kyowa-Z line and other type cultures were shown no growth inhibition mutually, but even in the case of *St. griseus* group, strains of *rhodochrous* SN and Kyowa-Z line were shown growth inhibition with each other.

It is an interesting result that the grisein-producing strains, *St. olivaceus*, *St. griseolus* and *St. scabis* were no growth inhibition with one another.

3. According to Dr. Krassilnikov's reports, the antagonisms (growth inhibition) with one another was a very suitable key for the classification of *Streptomyces*,

but authors have one opinion that the growth inhibition with one another of strains may be not stable key because of the qualitative variety of produced antibiotics and hardness of the acquisition of resistance.

Oxidative Metabolism of Phenylacetic Acid by *Penicillium chrysogenum* Q 176. (Addendum)

(p. *937～941*)

By Masao ISONO

(*Institute for Fermentation, Osaka*)

The author isolated many bacteria which can attack phenylacetic acid and use it as substrate for growth. The attack is in all cases brought about by adaptive enzymes. These bacteria divided into 4 groups owing to their mode of phenylacetic acid oxidation.

Cells of the 1st group grown on phenylacetate show complete simultaneous adaptation to *o*-hydroxyphenylacetic acid and homogentisic acid, but not to *m*-, *p*-hydroxyphenylacetic acid and homoprotocatechuic acid.

Cells of the 2nd group grown on phenylacetate show complete simultaneous adaptation to *o*-, *m*-hydroxyphenylacetic acid and homogentisic acid, but not to *p*-hydroxyphenylacetic acid and homoprotocatechuic acid.

Cells of the 3rd group grown on phenylacetate show complete simultaneous adaptation to *p*-hydroxyphenylacetic acid and homoprotocatechuic acid, but not to *o*-, *m*-hydroxyphenylacetic acid and homogentisic acid.

Cells of the 4th group grown on phenylacetate show complete simultaneous adaptation to *m*-, *p*-hydroxyphenylacetic acid and homoprotocatechuic acid, but not to *o*-hydroxyphenylacetic acid and homogentisic acid.

These facts suggest that the oxidative pathways of phenyl acetic acid by these bacteria may be formulated in the following sequences:

The 1st Group : Phenylacetic acid ⟶ *o*-Hydroxyphenylacetic acid ⟶ Homogentisic acid ⟶

$$\begin{tikzcd}[column sep=small, row sep=tiny] & o\text{-Hydroxyphenylacetic acid} \arrow[dr] & & \\ \text{The 2nd Group: Phenylacetic acid} \arrow[ur] \arrow[dr] & & \text{Homogentisic acid} \arrow[r] & {} \\ & m\text{-Hydroxyphenylacetic acid} \arrow[ur] & & \end{tikzcd}$$

The 3rd Group : Phenylacetic acid ⟶ *p*-Hydroxyphenylacetic acid ⟶ Homoprotocatechuic acid ⟶

$$\begin{tikzcd}[column sep=small, row sep=tiny] & m\text{-Hydroxyphenylacetic acid} \arrow[dr] & & \\ \text{The 4th Group : Phenylacetic acid} \arrow[ur] \arrow[dr] & & \text{Homoprotocatechuic acid} \arrow[r] & {} \\ & p\text{-Hydroxyphenylacetic acid} \arrow[ur] & & \end{tikzcd}$$

Studies on the Formation Mechanism of Black Tea Aroma. Part V. On the Precursor of the Volatile Carbonyl Compounds of Black Tea.

(p. *941～945*)

By Toshio NAKABAYASHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Shizuoka University*)

In order to make clear the precursor of volatile carbonyl compounds (VCC) of black tea, the withered tea leaves were ground with sugars, alcohols, and organic acids, and amino acids, fermented, and determined the increase of VCC.

As the results of these experiments, no VVC was produced from sugars, alcohols, and organic acids, but much VCC was determined from several amino acids. VCC produced from amino acids were as follows; acetaldehyde from alanine, aspartic acid, threonine, and cysteine, *n*-propion aldehyde from α-amino butylic acid, *i*-butyl aldehyde from valine, *i*-valer aldehyde from leucine, methyl ethyl acetaldehyde from isoleucine, phenyl acetaldehyde from phenyl alanine, and γ-methyl mercaptopropionaldehyde from methionine.

The VCC formation from the added amino acid was not produced by the unfermented leaves but by the fermented and boiled leaves and by the heating with the oxidized catechol by polyphenoloxidase.

This phenomenon was, therefore, the oxidative deamination and decarboxylation of amino acid by the action of quinoid type catechins in fermented tea leaves and a part of precursor of aldehyde in black tea aroma was amino acids produced in the withering time.

Studies on the Wax and Hydrocarbone in Sawamillet Bran Oil.

(p. *946～947*)

By Mitsuo KITAMURA and Tetsujiro OBARA

(*Department of Agricultural Chemistry, Tokyo University of Education*)

We have inspected on the wax which is insoluble in petrolium ether in Sawamillet bran oil.

Adding three times quantity of petrolium ether to 1.8 kg Sawamillet oil which is extracted with ethyl ether from Sawamillet bran (14 kg), we obtained 20.4 g yellow brown waxy substance.

This substance was treated with ligroin and fractionated about 43% wax fraction and about 32% hydrocarbone fraction with hot ethyl alcohol.

By saponifing the wax fraction we obtained cerylalcohol (H, 14.20; C, 81.39; m.p. 77–78°) from unsaponifiable matter and fatty acid which is considered as α-hydroxy-*n*-cosanic acid (H, 12.77; C, 74.41;

m.p. 95–96°; Neut. Val. 158.59) and cerotic acid (m.p. 74–75°; Neut. Val. 142.99) from saponifiable matter.

By recrystallizing the hydrocarbone with petrolium ether, we obtained nonacosane (H, 14.53; C, 84.99; M.W. 423.8; m.p. 63–64°) and a hydrocarbone which is considered as hentriacontane (H, 14.53; C, 84.75; M.W. 445.9; m.p. 68.5–69°).

Chromic Oxide Indicator Method for Digestibility Studies with Swine. (II) Accuracy of Digestibility determined by Indicator Method. (p. *947~951*)

By Shikao ISHIKAWA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Mie University*)

(1) The recovery of chromic oxide in feces was excellent in case of 1% of the indicator, when it was thoroughly mixed with the ground concentrate.

(2) The addition of chromic oxide to the ration had no effect on digestibility coefficients determined in conventional digestion trial.

(3) Variance analysis showed no significant differences among estimated coefficients based upon 11 grab samples for every organic nutrient. The relative standard error of estimated coefficients of crude fibre was greatest and that of crude protein smallest. In the experiment the mixture of approximately equal amounts of morning and afternoon excrements was assumed to be day grab sample and feces collected during night to be night one, although these were not true "grab" samples.

(4) It was concluded that digestibility data based upon grab samples agreed statistically with those determined in conventional 5-day digestion trials, althoug the former tended to be slightly lower than the latter.

Studies on the Amylase Formation by *Aspergillus* sp. Part VI. The Effects of Analogues of Amino Acids on α-Amylase Formation in *Aspergillus oryzae*. (p. *951~956*)

By Kenzo TONOMURA, Hideo SUZUKI, Keigo NISHINO* and Osamu TANABE

(*Fermentation Research Institute, Agency of Industrial Science and Technology, Chiba*)

As the previous study indicated that this α-amylase was an inducible enzyme and maltose or isomaltose was effective as an inducer, the present study was undertaken standing on this point of view. In this paper it was found that the following amino acid analogues, ethionine, glutamic acid-γ-hydrazide and norleucine prevented the α-amylase formation respectively, and their respective homologue amino acid reversed the prevention. It was also shown that both the extent of the prevention by ethionine and the effect of inducer were less in an old mycelium than in a young mycelium. The relationship between α-amylase formation and internal free glutamic acid which was estimated by isotope dilution analysis was discussed. From these results it was suggested that α-amylase formation might involve the internal free amino acids in case of a young mycelium at least.

* *The Student of Tokyo University of Agriculture and Technology.*

Chromatography of Organic Insecticides. Part I. Separation of Insecticides on Phenylcellosolve Treated Paper. (p. *956~960*)

By Takashi SHISHIDO and Masana SUWANAI

(*National Institute of Agricultural Sciences, Tokyo*)

Twenty-five organic chlorine and phosphorus insecticides are separated by partition between the more polar stationary organic solvent and the partially miscible, less polar, moving solvent. The paper is dipped in the stationary solvent diluted with acetone and the excess is pressed out between filter papers. After drying, the chromatograms are developed. An acetonitrile saturated *n*-hexane (mobile)/phenylcellosolve (PC)–acetone (1·5) (stationary) solvent system is suitable for the less polar chlorinated insecticides (BHC, DDT, cyclodiene compounds), while, for the separation of the phosphorus insecticides of relatively more polarity this solvent system is less satisfactory, as the R_F values are very low. *n*-Hexane–chloroform (or benzene)–acetonitrile (50:5·6)/PC-acetone (1:10 or 20) or PC-propyleneglycol–acetone (1:1:10 or 20) solvent systems are preferable for the separation of methyl parathion, ethyl parathion, isochlorthion, paraoxon, EPN, malathion, systox, metasystox, and gusathion. With decreasing the amount of the stationary phase on the paper the R_F values increase, but it too little, the mobile solvents dislodge the stationary phase. Dipterex having a hydroxyl group in the molecule is separated best in a more polar *n*-hexane–chloroform–acetic acid (10:20:1)/ethyleneglycol–acetone (1:10) solvent system.

Soil and Fertilizer, Inorganic Chemistry

M. NAKAYAMA: A New Basic Triple Salt Containing Magnesium Hydroxide. Part II. The Quaternary System $KCl–MgCl_2–Mg(OH)_2–H_2O$ at 100°. 46

Short Communications

K. MUNAKATA, T. SAITO, S. OGAWA and S. ISHII: Oryzanone, an Attractant of the Rice Stem Borer. 64

S. NAKAMURA and T. SHIRO: Hydroxyaspergillic Acid as Growth Inhibitant against Hiochi-bacteria (Studies on Growth Inhibition of Hiochi-bacteria, Specific Saprophytes of Saké. Part 4). 65

K. ANNO, N. SENO, E. NAKAMURA, H. SAITO and R. HOSHI: Isolation of Laminaribiose from Reversion Products of D-Glucose. 67

T. YAMAMOTO and J. FUKUMOTO: Reactivation of Bacterial α-Amylase Inactivated with Ethylenediamine Tetraacetate. 68

K. YAMADA, T. SUZUKI and M. TANAKA: Decomposition and Formation of Oxalate in Fish Skeletal Muscle. 71

Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI...... 1~10

PRINTED BY
KASAI PUBLISHING & PRINTING CO.
(Pan-Pacific Press)
MINATO-KU, TOKYO